PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/82, 15/84 — A1

(11) International Publication Number: **WO 98/13503**

(43) International Publication Date: 2 April 1998 (02.04.98)

(21) International Application Number: PCT/AU97/00625

(22) International Filing Date: 23 September 1997 (23.09.97)

(30) Priority Data:

08/717,971	23 September 1996 (23.09.96)	US
PO 2756	4 October 1996 (04.10.96)	AU
PO 5092	13 February 1997 (13.02.97)	AU
08/804,879	24 February 1997 (24.02.97)	US

(71) Applicant *(for all designated States except US)*: F.B. INVESTMENTS PTY. LTD. [AU/AU]; 825 Stanley Street, Woolloongabba, QLD 4102 (AU).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: TEASDALE, Robert, Dixon [AU/AU]; 33 Maculata Drive, Chapel Hill, QLD 4069 (AU).

(74) Agent: PIZZEYS PATENT AND TRADE MARK ATTORNEYS; Level 6, 444 Queen Street, Brisbane, QLD 4000 (AU).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: A PLANT AND METHOD OF MODIFICATION

(57) Abstract

A method of modifying a plant to increase vegetative growth of commercially valuable plant structures at the expense of non–essential and non–commercial structures is provided. From reproductive tissues of both *Pinus radiata* and *Eucalyptus grandis*, cDNA were isolated corresponding to genes which were specifically expressed during early development in both male and female plant structures. The promoter regions to these genes were isolated from respective genomic DNAs and fused to a structural gene for a bacterial ribonuclease, Barnase. In a variation of the method, an expression control system was used to control promoter leakage. Gene constructs were also prepared which expressed anti–sense forms of essential MADS box genes for these plants. Candidate gene constructs were used to transform shoot–forming tissues of target eucalypt and pine species. Explants from successful transformants were assessed for incorporation of the gene constructs and production of sterile plants.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaz land
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Paso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

A PLANT AND METHOD OF MODIFICATION

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of Application Ser. No.08/717971, a continuation of Ser. No.08/384208 (ABANDONED) filed 3 February, 1995, which was a continuation-in-part of Application Ser. No.08/210730 (ABANDONED) filed 18 March, 1994, which was a continuation-in-part of Application Ser. No.08/039419 (ABANDONED) filed 15 April, 1993, being the United States National Phase of PCT/AU91/00445 filed 26 September, 1991.

FIELD OF THE INVENTION

This invention relates to new plants and methods of modification thereof.

This invention has particular but not exclusive application to forestry and increasing the productive capacity of trees, and for illustrative purposes reference will be made to such application. However, it is to be understood that this invention could be used in other applications, such as the modification of other plants such as certain leafy food crops to increase the production of useful parts thereof.

BACKGROUND OF THE INVENTION

It is well recognized that the useful structures of domestic and commercial plants are in competition in the plant with other non-essential structures for growth resources such as water, nutrients and photosynthesis products. For example, the development of reproductive structures on forest trees represents a significant burden on the resources of the trees, the reproductive effort occurring at the expense of vegetative growth. The extent of the effect of reproductive burden on vegetative growth may be estimated by measuring the proportion of photo-assimilate which is directed to flowers and fruits, or strobili and cone growth for gymnosperms. In conifers,

reproductive effort is variously estimated to be up to 16% of annual photosynthate. The formation of reproductive structures imposes a burden on the tree beyond that of carbon allocation. First, these structures have very large requirements for important nutrients at the expense of vegetative tissues so that, at least under conditions of nutrient deficiency, prevention of reproductive structure formations as proposed can lead to a greater increment in vegetative growth than the corresponding mass of reproductive structure otherwise formed.

Nutrient losses from pollen dispersal are not inconsiderable in most forest plantations, with an estimated annual production of 370 kg/ha of nutrient-rich pollen by Pinus radiata plantations. Tree crop productivity of P. radiata is as yet only about 50% of that theoretically obtainable on the basis of incident solar radiation. Therefore, in many environments, there are good prospects for significant increase in harvest yield from trees which have been inhibited from producing reproductive structures.

It is noted that there is a deleterious effect in at least P. radiata on the value of timber caused by persistent stem cones, and research has been advocated for methods for chemically inducing cone abscission in species such as P. radiata. However, it is clear that means involving chemical treatment of forest trees will involve considerable expense in manufacture and application, this cost being exacerbated in certain circumstances where environmental factors both preclude indiscriminate broadcast of such chemicals and insist on strict control of run off.

Seeds from mature trees will produce an undergrowth of saplings which must be removed periodically to prevent competition with the tree crop for soil resources and to minimize

the risk of damaging fire. This sapling removal is labour intensive, time consuming and accordingly expensive, adding considerably to the production cost of farmed forest timber. Trees and other plants may form reproductive structures at their apical meristem, leading to unwanted termination of vegetative growth at this apice. Such terminal flowering may effect the form of trees, with heavier branching which reduce the quantity and value of harvestable timber if the trees did not continue to grow vegetatively.

US Patent No. 5122466 discloses the transformation of Loblolly pine by bombardment with microprojectiles carrying an expression cassette to yield transformants. The disclosure provides a method establishing that a plasmid bearing a functional gene construct can successfully transform plant cells. However, the disclosure does not provide for inducing sterility in plants.

Koltunow et al. (The Plant Cell, Vol2, 1201-1224) discloses methods of inducing male sterility in plants by expression of a lethal gene (Barnase) in plant stamen cells (tapetal). A chimeric gene comprising the Barnase gene inserted into and under the expression control of the TA29 gene of tobacco, a gene established to be specifically expressed in tapetal tissue. The disclosure relates to the induction of sterility only, but does not overcome the problem of plants developing reproductive structures at the expense of vegetative growth.

The present invention aims to alleviate the above disadvantages and to provide plants of enhanced productive capacity and methods for the production thereof which will be reliable and efficient in use. Other objects and advantages of this invention will hereinafter become apparent.

SUMMARY OF THE INVENTION

With the foregoing and other objects in view, this invention in one aspect resides broadly in a method of enhancing vegetative growth in a plant including the steps of:-

identifying a gene having a substantially tissue-specific promoter expressing during the development of both male and female plant reproductive structures;

constructing an expression cassette comprising a heterologous coding region capable of expressing a product which aborts said development under the expression control of said promoter;

transforming plant cells with said expression cassette, and selecting and vegetatively propagating the transformants.

Preferably, the non essential structure is selected from the reproductive structures of plants which are economically capable of artificial vegetative propagation. Preferably, the plant selected is a tree for timber, pulp or fibre production wherein poor or absent expression of reproductive structures may result in increase in vegetative growth of the valuable material.

A large number of genes are differentially expressed between sexual and vegetative buds. Accordingly, it is preferred that the gene be selected from those specific to sexual budding, specific to the production of other sexual structures, or specifically coding for a product essential in a developmental pathway for a reproductive structure. The gene is preferably selected for its early expression, specifically in the developing reproductive tissues.

For example, five cone-specific genes displaying strong homology to *Arabidopsis thaliana* and *Anthirrhinum majus* floral meristem and organ identity genes have been identified in *P.radiata* cDNA library prepared from immature female and male

cones. Three of them, PrMADS1, 2 and 3, belong to the family of MADS-box genes showing homology to *Arabidopsis AGL-2, AGL-4* and *AGL6* genes and *dal1* gene from another non-angiosperm, *Picea abies* (Norway spruce), respectively. The PrFL1 gene is the pine ortholog of *Arabidopsis Leafy* (*Lfy*) and *Floricaula* (*Flo*) gene from *Anthirrhinum*. The *PrCon1* shows strong homology to *Arabidopsis* CONSTANS (CO) gene. A significantly lower level of expression was detected in vegetative tissues: vegetative buds, needles, stem and roots. *In situ* hybridisation showed that expression of these genes is substantially detectable only in reproductive tissue cells.

Expression analysis has revealed that all five genes show different patterns of expression in different stages of development of male and female cones. PrMADS1, 2 and 3 genes are cone-specific in that expression of both genes was substantially restricted to reproductive organ primordium tissues. No detectable expression of these genes was observed in vegetative tissues such as vegetative buds, needles, stems, roots. For PrFL1 and PrCon1 low detectable expression was observed in vegetative buds.

In reproductive organs low level of expression of both genes was detected at early stages of cone development (5 mg cones) which was increased during a cones development (50 mg cones). In male cones expression of both genes was restricted to microsporangium containing primary sporogenous cells. In female cones expression of both genes was restricted to premature ovules.

In a further example, MADS-box genes of *Eucalyptus spp.* have been identified as having the reproductive organ specificity required of the present invention and identified hereinafter as genes EGM1, 2 and 3 respectively, gene EGM3 being highly specific

for reproductive primordia.

Preferably, the gene is identified in cDNA libraries prepared from mRNA isolated from reproductive tissues and selected by differential screening against mRNA of vegetative structures. Since the biochemical pathways for vegetative buds and developing reproductive structures may include common expression products and consequently similar mRNAs, it is preferred to differentially screen cDNA libraries against vegetative bud mRNA, with or without preceding enrichment of the cDNA for genes specifically expressed in reproductive tissues. The selected gene may be any which when its expression is blocked or otherwise made ineffectual, results in the failure to produce a non essential plant structure.

Alternatively, the cDNAs may be utilized as probes to select corresponding genomic clones from genomic libraries. The genomic clones may be used to isolate and identify gene promoters that specifically express genes unique to reproductive structures. Such promoters can then be combined with lethal genes which when expressed will inhibit or terminate growth of the cells within which the lethal gene is expressed. The promoter-gene fusions when stably incorporated into a plant, by any suitable known means, will result in failure to develop reproductive tissues in whole or in part.

Candidate cDNA clones may be raised and are preferably selected for the presence of genes expressed in both male and female buds. The selection may be directed to those genes which are naturally produced or those which are induced by plant growth regulators which promote flowering, such as various gibberellins. Preferably, the specific expression in floral tissues is confirmed using an in situ RNA hybridization procedure with a wide range of different plant tissues.

The preferred cDNA clones are those further selected for the characteristics of early appearance and highly specific expression.

The modification of the identified gene may be by fusing the tissue-specific promoter of said gene with a structural gene for a deleterious or lethal product such that regenerated plants transformed with said gene-fusion will not form said non-essential structure. Alternatively, a critical function of said gene may be disrupted or modified by expression of the modified gene in transformed plants.

The modified gene is preferably introduced into a plant normally containing the identified gene such that a critical function of the identified gene is disrupted or modified. However, it is also envisaged that introduction of the modified gene into plants not containing the specifically identified gene may result in useful reduction or elimination of a non-essential plant structure, particularly where the gene selected has analogues represented across several species, or in closely related species where the corresponding gene is essentially homologous with the gene in question.

Modification of the gene may be achieved by any suitable means, with the expression strategy desired being the primary arbiter of the modification process utilized. For example, it may be intended to constitutively express an antisense or perhaps ribozyme version of a gene which is critical to development of reproductive structures, so that the normal gene action is disrupted and vegetative development occurs instead. The method may be to splice a promoter specific to a reproductive structure with a lethal gene which codes for an expression product which will cause abortion of the tissue in which it is produced, for example, buds that differentiate as reproductive structures.

Genes expr ssing antisense RNA against the mRNA coded by each of the selected target genes may be constructed. These and shorter RNA sequences that bind to the initiation regions of the target mRNA may be used for inhibition of translation. Of course, other critical regions apart from the translation initiation site may be targeted for binding of antisense RNA.

One option is to use an antisense or ribozyme version of a critical house-keeping gene, such as the actin gene or a gene coding for an enzyme of aromatic amino acid biosynthesis, for example enolpyruvyl shikimate phosphate synthase. Alternatively, a deleterious enzyme such as a protease, ribonuclease, or deoxyribonuclease may be encoded for biosynthesis under control of a sexual promoter or any promoter specifically expressed in a non essential structure.

One difficulty with the tissue ablation approach is the effect of promoter leakage or expression in other tissues. Accordingly, where the promoter controlling the heterologous coding region in expression is merely substantially tissue specific, any residual leakage is preferably overcome by modification of the expression cassette to include appropriate leakage control. For example, to overcome leakage a second copy of the substantially tissue specific promoter, or a second tissue specific promoter, may be used to promote a gene producing a control product capable of switching on production of an inhibitor to the lethal gene product in non target tissues. One example of this approach is to construct an expression cassette including a control system (gene cascade) whereby expression of a lethal gene(s) is countered in non-target tissues, such as cassettes carrying both Barnase and Barstar where Barstar is under the constitutive control of a promoter responsive to repressor *lacIq*, itself promoted by a second copy of the selected

tissue specific promoter.

In the case of *Eucalyptus* transformation and regeneration, there are particular features of the species which have required the development of specific transformation protocols. Accordingly, in a further aspect this invention relates to a novel method for *Agrobacterium*-mediated transformation of *Eucalyptus* shoot and seedling explant tissue and regeneration of plants. *Eucalyptus* species are known for their fast growth and high biomass productivity. They are grown in commercial forestry plantations around the world. Reforestation, the controlled regeneration of forests, has become an integral part of forest management in order to secure a renewable and sustainable source of raw material for production of paper and other wood-related products. Forest trees can be regenerated by either sexual or asexual propagation. Sexual reproduction of seedlings for reforestation has traditionally been the most important means of propagation. Regeneration by seed usually results in highly variable progeny. On the other hand, vegetatively propagated clonal planting stock have a number of potential benefits including uniformity in quality and growth.

Eucalypts are primarily native trees to Australia with a small number of exceptions in the Asian region. However, they are now widely grown around the world. Several species of *Eucalyptus* are fast growing trees of importance for timber and paper pulp industries. Genetic improvement of eucalypts, as of other trees, is difficult with traditional breeding because of the constraint imposed by long developmental cycles.

During the last two decades, substantial progress has been made in genetic engineering of plants. However, progress in the application of biotechnology to trees has been slow and there have been only a few reports concerning *Eucalyptus* (Teulieres *et*

al., 1994). Assessment of these reports is difficult as the technical details of these reports are generally not available. However, these studies have concentrated on the good regeneration properties of *E. camaldulensis* seedling explant tissue. The main difficulties for Eucalyptus transformation appeared to be in establishment of suitable regeneration systems as well as the low transformation frequencies. Biolistic bombardment has been tested as an alternative to *Agrobacterium*-mediated transformation, although transient expression was observed (Rocharge *et al.*, 1995), transformed *Eucalyptus* plants were not regenerated. Extensive work has been performed on transformation through electroporation of protoplasts (Teulieres *et al.*, 1994). However, there has been only one report concerning successful transformation of *Eucalyptus* protoplasts (Kawasu *et al*, 1991). There are no reports on the transformation and regeneration of *Eucalyptus* shoot material. Shoot explants are generally used for the micropropagation of elite clones.

There is an urgent need to increase the quantity and quality of fast growing timbers in the future and the micropropagation of elite transgenic *Eucalyptus* can play a significant role.

It is therefore, desirable to provide a method for the introduction of genes into *Eucalyptus* species for mass production of clones of genetically modified and improved trees. It is further desirable to provide a multistage regeneration protocol which can be utilised effectively on *Eucalyptus* species to produce large quantities of plants for field planting. It is further desirable to provide a progression of steps which, in combination, provide a method for transformation and regeneration of diverse genotypes and species of *Eucalyptus* and *Eucalyptus* interspecies hybrids. In addition, it is desirable to combine gains from conventional genetic testing and breeding with

mol cular genetic improvement to produce a high value-added *Eucalyptus* plant.

These objects and others are addressed by the present invention, which is designed to produce elite *Eucalyptus* plants. These objectives are achieved by a multi-step method for the regeneration of *Eucalyptus* plants. Although other transformation and regeneration protocols have been published, none of these methods have proven totally effective with the *Eucalyptus* species in that none enabled the practitioner to reliably proceed from the beginning step of shoot explant transformation to completion of the regeneration process resulting in establishment of plants in field conditions. The present protocols provide such a multi-step method for *Eucalyptus* plants. It incorporates two key steps. The first is keeping the shoot explant intact and upright during the co-cultivation step with *Agrobacterium* so that the leaves do not touch the tissue culture medium. The second key step is the incorporation of a liquid culture selection stage. This liquid culture selection step is only effective once regenerated shoots have been produced on solid medium. Shoot material grew faster in liquid medium and formed more shoots when compared to shoots grown on solid medium. This liquid selection step has the advantages of reducing false positives by increasing selection pressure, reducing residual *Agrobacterium* and increasing the amount of shoot tissue compared to solid grown cultures.

Typically, the process may proceed according to the following steps:

1. The *Agrobacterium*-mediated transformation of *Eucalyptus* explant material with DNA. The source of the explant material may be from seedlings or micropropagated clonal plant tissue.

2. The *Agrobacterium* and explant are co-cultivated. In the case of shoot explants, the shoots are kept upright in the

solidified medium so that the leaves are not in physical contact with the medium.

3. The explants are then transferred to medium containing an antibiotic which kills the *Agrobacterium*. This selection is maintained for the remainder of the plant regeneration procedure.

4. The explants are then transferred to callus induction medium containing a selective agent which only allows the growth of tissue containing the introduced DNA. This selection is also maintained for the remainder of the plant regeneration procedure.

5. The explants are then transferred to shoot induction medium.

6. The regenerated shoots are then put into in liquid culture.

7. Surviving shoots and callus are transferred to solid medium.

8. Putative transformed shoots are assayed for presence of the introduced DNA.

9. Transgenic shoot material is then transferred to root induction medium.

10. After root elongation and development, the plantlets are transferred to soil or other growing medium in a container where root development and acclimatisation (gradually lowering relative humidity) continue.

12. Clones can be micropropagated by tissue culture propagation techniques and grown into trees of a size and form suitable for planting.

There are several advantages inherent with the use of this novel method. For example, the method is well suited for large-sc le production of clones of g netically modified and

improved *Eucalyptus*. Th present processes also provides a reliable multi-step regeneration method for the recalcitrant *Eucalyptus* species. It is the combined application of the progression of steps in this novel multi-step method that has enabled the first successful field planting of many different genotypes of *Eucalyptus*.

Apart from the use of infective agents such as *Agrobacterium* for transformation of pines and eucalypts, it has now been determined that new protocols for transformation by biolistic bombardment are useful for pines. Transformation of conifers generally requires embryogenic cultures as target cells. The advantage of using such cultures is that they offer a uniform and fast growing population of cells which can develop into embryoids from single cells.

It has now been determined that transformation of pine and particularly *Pinus radiata* can be achieved using mature embryos and shoot forming callus as targets.

Using the observation that the shoot meristem of pine is different from its counterpart of dicots and monocots in that it does not have a multiple layer structure and is comprised of a single type of meristematic cell capable of dividing in any direction so as to form a meristematic mass, it has been established that biolistic bombardment is a realistic alternative transformation strategy for such species. Shoot forming callus of pine has a similar structure to shoot meristem and is amenable to this manner of transformation also.

In the light of the foregoing, there has been established a tissue culture system which regenerates shoots from mature embryos of radiata pine at a consistently high frequency. Shoot forming callus may be induced from shoot tips and bases

of young needles.

The invention will be further described with reference to the following Examples and Figures supporting, illustrating embodiments of the present invention, and wherein:

FIG 1 is expression of the PrMADS1, PrMADS2, PrMADS3, PrFL1 and PrCON1 genes in reproductive and vegetative tissues of *P.radiata*;

FIG 2 is expression of PrMADS1 gene in female cones;

FIG 3 is expression of PrMADS2 gene in female cones;

FIG 4 is expression of PrMADS2 gene in male cones;

FIG 5 is expression of PrMADS3 gene in male and female cones;

FIG 6 is gel mobility shift assay of PrMADS1, PrMADS2 and PrMADS3;

FIG 7 is a promoter finder strategy;

FIG 8 is the nucleotide sequence of PrMADS1 cDNA clone;

FIG 9 is the amino acid sequence of PrMADS1 protein;

FIG 10 is the nucleotide sequence of PrMADS2 cDNA clone;

FIG 11 is the amino acid sequence of PrMADS2 protein;

FIG 12 is the nucleotide sequence of PrMADS2 promoter;

FIG 13 is the nucleotide sequence of PrMADS3 cDNA clone;

FIG 14 is the amino acid sequence of PrMADS3 protein;

FIG 15 is the nucleotide sequence of PrMADS3 promoter;

FIG 16 is the nucleotide sequence of PrFL1 cDNA clone;

FIG 17 is the amino acid sequence of PrFL1 protein;

FIG 18 is the nucleotide sequence of PrFL1 promoter;

FIG 19 is the nucleotide sequence of PrCon1 gene (partial);

FIG 20 is a diagrammatic illustration of a control mechanism to protect non target tissues;

FIG 21 is the plasmid pBR Barnase prom Barstar 1

containing V1-promoters;

FIG 21A is a diagrammatic illustration of plasmid 7 prom Barstar comprising a promoter and barstar fragment cloned into pGem3zf (Promega);

FIG 22 is a diagrammatic illustration of plasmid containing V2-promoters;

FIG 24 is a Northern blot of total RNA extracted from Eucalypt tissue probed with the EGM2 cDNA (3 day exposure);

FIG 25 is a graphical representation of Northern blot of total RNA extracted from Eucalypt tissue probed with the EGM2 and EGM3 cDNA respectively (6 hour exposure);

FIG 26 is a Northern blot of total RNA extracted from Eucalypt tissue probed with the EGM3 cDNA (6 hour exposure);

FIG 28 is the nucleotide sequence of the EGM1 promoter;

FIG 28A is a diagrammatic illustration of the EGM1 promoter;

FIG 29 is the nucleotide sequence of the EGM3 promoter;

FIG 29A is a diagrammatic representation of the EGM3 promoter;

FIG 29B is the nucleotide sequence of the EGM3 promoter;

FIG 30 is the nucleotide sequence of the EGM2 promoter;

FIG 30A is a diagrammatic representation of the EGM2 promoter;

FIG 31 is a diagrammatic representation of agarose separation of a *PstI* digest of an EGM3 double bin. Given that the *EcoRI* cloning site is at base 6772 in EGM3 bin, the predicted fragment sizes from the map for this digestion are 4.9, 4.8, 4.4, 3.3, 1.9, 1.1, and 0.6 kb;

FIG 32 is a diagrammatic representation of two gel exposures of a *PstI* digest of an EGM3 bin showing the other possible orientations of the cloned insert. The sizes of fragments expected from this digest are 7.4, 4.9, 4.4, 1.1, 0.7, and 0.6 kb;

FIG 33 is a plasmid diagram of EGM3 double bin wherein the illustrated fragment is cloned in both orientations;

FIG 34 is a plasmid diagram of EGM3 V1 sense;

FIG 35 is a plasmid diagram of plasmid V1 (Barnase-

Barstar);

FIG 36 is a plasmid diagram of pBR Barnase;

FIG 37 is a plasmid diagram of plasmid V2 (LacIqNLS-35S promoterOp-Barstar);

FIG 38 is a plasmid diagram of p35Sop Barstar E-2;

FIG 39 is a plasmid diagram of p35Sop Barstar, and serves also to represent 35Sop Barstar E, which is the same plasmid with the *EcoRI* site at base508 removed;

FIG 40 is a plasmid diagram of pBRlac;

FIG 41 is a plasmid diagram of pBRGUS 1;

FIG 42 is a plasmid diagram of pBRGUS 2;

FIG 43 is a plasmid diagram of Bin 19+EGM3 V1 sense (Orientation 2 as described in the following description of embodiments);

FIG 44 is a plasmid diagram of EGM3 V2 sense;

FIG 45 is a plasmid diagram of plasmid pBRLacBH-;

FIG 46 is an illustration of *Arabidopsis* (FB.13L30) transformed with an antisense PrMADS1 gene compared with a control plant; and

FIG 47 is a diagrammatic representation of the directed development strategy resulting in the transformed *Arabidopsis* of FIG 46.

EXAMPLE 1

Isolation of cone-specific genes from *P. radiata*

Five cone-specific genes displaying strong homology to

Arabidopsis thaliana and *Anthirrhinum majus* floral meristem and organ identity genes were isolated from *P.radiata* cDNA library prepared from immature female and male cones. Three of them, PrMADS1, 2 and 3, belong to the family of MADS-box genes showing homology to *Arabidopsis AGL-2, AGL-4* and *AGL6* genes and *dal1* gene from another non-angiosperm, *Picea abies* (Norway spruce), respectively. The PrFL1 gene is the pine ortholog of *Arabidopsis Leafy* (*Lfy*) and *Floricaula* (*Flo*) gene from *Anthirrhinum*. The *PrCon1* shows strong homology to *Arabidopsis CONSTANS* (*co*) gene. To elucidate the function of these genes the approach of characterising their expression pattern in male and female cones during different stages of cone development was taken.

A significantly lower level of expression was detected in vegetative tissues: vegetative buds, needles, stem and roots. *In situ* hybridisation showed that expression of these genes is substantially detectable only in reproductive tissue cells.

Expression analysis revealed that all five genes show different patterns of expression in different stages of development of male and female cones (Figs. 1-5). PrMADS1, 2 and 3 genes are cone-specific: expression of both genes was restricted to reproductive organ primordia tissues. No detectable expression of these genes was observed in vegetative tissues: vegetative buds, needles, stems, roots. For PrFL1 and PrCon1 low detectable expression was observed in vegetative buds.

In reproductive organs low level of expression of both genes was detected at early stages of cone development (5 mg cones) which was increased during a cones development (50 mg cones). In male cones expression of both genes was restricted to microsporangia containing primary sporogenous cells. In female cones expression of both genes was restricted to premature ovules.

To characterise MADS proteins as DNA-binding proteins *in*

vitro, we expressed both proteins in *E.coli* and characterised their DNA-binding properties. PrMADS1,2,3 proteins are sequence-specific DNA-binding proteins. Their DNA-binding consensus sequence is similar to that of the *AGAMOUS* protein. All three proteins bind a DNA sequence matching the consensus sequence of CArG box TT(A/T)CC(A/T)(A/t)$_2$(T/A)NNGG(-G)(A/T)$_2$ (oligo A) for PrMADS1 and PrMADS2) (Fig.6). Mutation of these consensus sequences (oligo B) significantly decreases their binding of PrMADS 1,2, or 3 proteins. Competition with non-radioactive oligos did not decrease binding of any of the proteins to the CArG consensus. This indicates that we are dealing with specific DNA-protein interactions.

EXAMPLE 2

Isolation of promoter sequences of PrMADS2,3 and PrFL1 genes

Upstream sequences were isolated using a 'Promoter finder' strategy (Fig.7). A special adaptor was ligated to the ends of DNA fragments generated by digestion of genomic DNA from *P. radiata* with EcoRV, ScaI, DraI, PvuII and SspI separately. The enzymes used were selected because they have six-base recognition sites and generate blunt ends. Following adaptor ligation, these DNA fragments were used as a template for PCR using first adaptor primers AP1, AP2 and gene-specific primers GSP-1,2.

The sequences of adaptor (first sequence), adaptor-primers, and polymerase blocking primer are shown below. It is noted that adaptor primer AP1 corresonds to bases 1 to 22 of the adaptor, adaptor primer AP2 corresponds to bases 13 to 31, and the polymerase block corresponds to bases 41 to 48.

Adaptor: 5'GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3'

Polymerase block: 3'-NH_2-CCCGACCA-PO_4-5'

Adaptor primer 1 (AP1): 5'-GTAATACGACTCACTATAGGGC-3'

Adaptor primer 2 (AP2): 5'-ACTATAGGGCACGCGTGGT-3'

The presence of the amine group on the 3' end of the lower strand blocks polymerase catalysed extension from free adaptor molecules that have not been ligated, thus preventing the generation of the primer binding site unless a defined, gene specific primer extends a DNA strand opposite the upper strand of the adaptor.

Primary PCR was performed using Avantage Tth Polymerase Mix (Clontech) and adaptor primer AP1 and GSP-1 for PrMADS2,3 and PrFL1 genes.

GSP-1 sequences:

PrMADS2: 5' CGGCGCTTCCGAACTCATAGAGTTTTCCTC 3'
PrMADS3: 5'TTAGCGCCACTTCGGCATCGCACAGC 3'
PrFL1: 5' CAAGGGACTTCAAATCCTTTCTCCCATTCATGG 3'.

PCR two step cycle parameters:

7 cycles:	94°C	25 sec
	72°C	4 min
32 cycles:	94°C	25 sec
	67°C	4 min

67°C for an additional 4 min after the final cycle.

The 1 ml of primary PCR was used in secondary PCR using the same cycling parameters and a second set of GSP primers with AP2 adaptor-primer.

GSP-2 primers sequences:

PrMADS2: 5' CGCCTTTTTCAGCAGACCATTCCGGC 3'
PRMADS3: 5' CAGCAGTCCGTTTCCGGCGCTTCG 3'
PrFL1: 5' CGTCCATGGTCCTTGTTAAAGACAGTTGTTGTTGG 3'

The 1-2 kb PCR fragments were cloned into the TA-type cloning vector and sequenced. Sequences of cDNAs, proteins and promoter regions PrMADS2, PrMADS3 and PrFL1 are shown on Figs 12, 15 and 18.

EXAMPLE 3

Introduction of promoter regions into the sterility cassettes V1 and V2

One difficulty with the tissue ablation approach is the effect of promoter leakage or expression in other tissues. To overcome this we have developed a control system (gene cascade) whereby expression of a lethal gene(s) is countered in non-target tissues (Fig.20). Two different vectors carrying Barnase (V1) and Barstar plus repressor (*lacIq*) (V2) parts of these cascade were designed.

In order to clone promoter regions into the vectors V1 and V2 they have been amplified using sets of primers with *Sal*I restriction sites on the ends for V1 (*Sal*I primers) and *Eco*RI restriction site for V2 (*Eco*RI primers). In order to check the orientation of the promoters in V1 and V2 vectors an *Eco*RI restriction site was designed in the forward *Sal*I primer downstream from *Sal*I site and a *Sal*I site was designed in the *Eco*RI primer downstream from *Eco*RI:

SalI primers:

PrMADS2 promoter:

Forward primer:

5' CCGGTCGACGAATTCCGACAGTGGAGTCCACAAAGAAAGATGCG3'

SalI *EcoRI*

Reverse primer: 5' CCGGTCGACTTCTTTCCTTCTTTTCTTTCTGC 3'

SalI

PrMADS3 promoter:

Forward primer: 5' ACGCGTCGACGAATTCAAGATTTCAAATCAGTCC 3'

SalI *EcoRI*

Reverse primer: 5' ACGCGTCGACCAAGATCCCTCTGCTTCTTCACC 3'

SalI

PrFL1 promoter:

Forward primer: 5' ACGCGTCGACGAATTCGAACTTCTGGAATAAGCTGC 3'

SalI *EcoRI*

Reverse primer: 5' ACGCGTCGACTTCATCTTACGTCACGCGAGG 3'

SalI

***EcoRI* primers:**

PrMADS2 promoter:

Forward primer:

5' CCGGAATTCGTCGACCGACAGTGGAGTCCACAAAGAAAGATGCG3'

EcoRI *SalI*

Reverse primer: 5' CCGGAATTCTTCTTTCCTTCTTTTCTTTCTGC 3'

EcoRI

PrMADS3 promoter:

Forward primer: 5' CGGAATTCGTCGACAAGATTTCAAATCAGTCC 3'

EcoRI SalI

Reverse primer: 5' GCGAATTCCAAGATCCCTCTGCTTCTTCACC 3'

EcoRI

PrFL1 promoter:

Forward primer: 5' CGGAATTCGTCGACGAACTTCTGGAATAAGCTGC 3'

EcoRI SalI

Reverse primer: 5' GCGAATTCTTCATCTTACGTCACGCGAGG 3'

EcoRI

PCR fragments of *SalI* primers were digested with *SalI* restriction enzyme and introduced into the V1 vector digested with *SalI*. The orientation of promoters was checked using digestion with *EcoRI* enzyme.

PCR fragments of EcoRI primers were digested with *EcoRI* restriction enzyme and introduced into the V1 vector digested with *EcoRI*. The orientation of promoters was checked using digestion with *SalI* enzyme. Maps of the resulting plasmids are shown in Figs. 21 and 22.

These vectors were linearised with HindIII enzyme and introduced into the Bin19 binary vector digested with HindIII. Colonies carrying modified Bin 19 vectors were selected on plates with kanamycin and ampicillin antibiotics. As a next step Bin19-V1-promoter and Bin19 V2-promoter vectors were transformed into several

Agrobacterium strains. *Arabidopsis thaliana*, *Eucalyptus grandis* and *Pinus radiata* embryos and explants were co-transformed with Agrobacterium strains.

EXAMPLE 4

Plasmid construction for lethal gene constructs

The HindIII fragment from the plasmid pRT 99 gus (Töpfer et al. Nucleic Acids Research (1988) 16 (17): 8725) was cloned into the HindIII site of pBR 322. This insertion resulted in two plasmids corresponding to the insert being cloned in both orientations. The inserted region contains the Cauliflower Mosaic Virus (CaMV) 35S RNA promoter, beta glucoronidase (GUS) gene, and CaMV 35S terminator region. These plasmids were called pBrGUS1 and pBrGUS2 and provided the basis for the lethal gene constructs. The orientation of the insertion event was checked with a BamHI digest. PBRGUS 2 results in BamHI fragments of 2.5kb and 4.4kb, pBRGUS 1 in fragments of 6.1kb and 0.8kb.

The DNA encoding the lacIq nuclear localisation signal peptide was amplified from the plasmid pGEM lacIqNLS by PCR using the 5' primer Lac I (this has an EcoR1 site) and the 3' primer D23 which has a KpnI site. The amplified DNA fragment was restricted with EcoRI and KpnI and cloned into pBRGUS 2 cut with the same enzymes. The resulting plasmid was called pBRLac. The pBRLac plasmid was then cut with SphI and run on an agarose gel to allow the purification of the plasmid fragment containing the LacIq gene, Ampicillin resistance gene, and origin of replication away from the small SphI fragment, which contained restriction sites that we wished to remove. The resulting plasmid was called pBRLacBH-.

The second stage in the plasmid construction was the preparation for cloning of the Barstar gene under the regulation

of the modified CaMV plus lac operator promoter. This was amplified from the plasmid p35S-op-Barstar EcoRI- which contained the promoter/gene sequence which had been modified to remove the EcoRI site between the promoter and coding sequence. This modification was accomplished by cutting the 35S-op-Barstar plasmid with EcoRI, carrying out a blunting reaction with T4 DNA polymerase, and then religating the blunted plasmid. The PCR was carried out using the 5' primer pQE-F and the 3' primer Bar-3'. The PCR fragment was cut with XhoI and KpnI. This was cloned into the 35S-op-Barstar EcoRI- plasmid also cut with XhoI and KpnI, and from which the unwanted gene had been purified away by gel electrophoresis. This enabled the removal of restriction sites at the three prime end of the Barstar coding region. This plasmid was called p35S-op-Barstar EcoRI- 2.

The Barstar gene was then cut out of the p35S-op-Barstar EcoRI- 2 plasmid with XhoI and SalI and cloned into pBrLac BH- cut with SalI. The Barstar gene could go into the SalI site in either orientation but only one orientation was found. The orientation of the insert was ascertained by a KpnI digest. Only a 1kb band was seen which was indicative of the orientation of the insert seen in plasmid pBRLac Op Barstar 1. The resulting plasmid was called V2.

The plasmid was then ready for the cloning of, in this case, a flower specific promoter, into the unique EcoRI cloning site 5 prime of the lacIq gene. The EGM3 promoter from the Eucalyptus MADS gene EGM3 was used for this purpose. This was cut from plasmid pEGM3 with EcoRI. This promoter was cloned in both orientations, and the resulting plasmids were called V2 EGM3 sense and V2 EGM3 antisense. The plasmid V2 EGM3 sense was used as a source the EGM3 regulated lacIq and CaMVop Barstar genes for the plant transformation vector. The orientation of the EGM3 promoter

was determined by an XbaI PstI digest. The presence of a 2.3 and 0.9 kb band was indicative of the correct orientation.

The second construct V1, containing the promoterless Barnase gene, was constructed using pBrGUS 2 as the starting point. A Barnase gene was amplified from genomic *Bacillus amyloliquefaciens* using the primers Barnase 5 prime Sal, and Barnase 3 prime Kpn. The resulting PCR product was restricted with Kpn I and Sal I as was pBRGUS 1 and a ligation reaction performed. The plasmids from the resulting colonies were used as templates for sequencing using the amplification primers. Plasmid SB4 was found to contain a Barnase fragment of the same sequence as the *B. amyloliquefaciens* Barnase gene and this was called pBRBarnase and this was used for further constructions.

Previous work using Barnase as a lethal gene in plants had shown that the anti-toxin gene for Barstar was required to be present and expressed from the same plasmid as the Barnase before a promoter region could be cloned 5 prime of the Barnase gene (Paul *et al.* 1992 Plant Molecular Biology 19: 611-622). This was achieved by using a plasmid that contained the Barstar gene and promoter from *B. amyloliquefaciens* in cis with the Barnase sequence. To this end the promoter plus Barstar DNA region was amplified from the *B. amyloliquefaciens* genomic DNA using primers 5 prime Barstar promoter and Bar 3 prime. The PCR fragment was restricted with KpnI as was pGEM 3f and a ligation reaction performed. The resulting white colonies were screened for inserts. The DNA from colony 7 was used for sequencing and found to contain the Barstar promoter plus Barstar sequence as in the genomic *B. amyloliquefaciens* DNA. This plasmid was call 7 prom Barstar, as shown in Fig 21A.

The 7 prom Barstar plasmid was restricted with Kpn I as was pBRBarnase and the Promoter Barstar fragment was cloned into the

Kpn I site of the pBRBarnase DNA. This resulted in a plasmid known as V1. The EGM3 promoter was cut out of pEGM3 using EcoRI, blunted using DNA polymerase I (Klenow fragment) and cloned into the unique SalI cloning site of the V1 DNA which had also been restricted and blunted. The resulting plasmid with the promoter inserted in the correct orientation was called EGM3 VI sense.

The EGM3 V1 sense DNA was linearised with HindIII and cloned into the plant transformation vector Bin19 also linearised with HindIII. This resulted in two plasmids, EGM3 V1 Bin 1 and 2, corresponding to the two orientations of insertion. In orientation 2, the two EcoRI sites present in EGM3 V1 Bin are approximately 80 bp apart. The EGM3 V1 Bin 2 was cut with EcoRI and blunted using DNA polymerase I (Klenow fragment). The EGM3 promoter lacIq gene, and the CaMV 35S op Barstar genes were cut out of EGM3 V2 sense using AatII and HindIII. This fragment was also blunted and ligated into the EcoRI cut and blunted EGM3 V1 Bin 2 to create the plasmids EGM3 Double Bin I and II, again corresponding to the two possible orientations. These plasmids were used for transformation of plasmids.

The procedure was then repeated with further Eucalyptus MADS promoters. These were EGM2 long and short, and a shorter version of EGM3.

Primers.

Barnase 5 prime Sal

5' CCGTCGACATGGCACAGGTTATCAA 3'

SalI

Barnase 3 prime Kpn

5' CGGGTACCTTATCTGATTTTTGTAAAGG 3'

KpnI

Five prime Barstar promoter

5' CGGGTACCGTCCAATCTGCAGCCGTCCGA 3'

KpnI

Bar 3'

5' GCGGTACCTTAAGAAAGTATGATGGTG 3'

KpnI

D23

5' CGGGTACCTATCTCCTATTTCCCCCACG 3'

KpnI

pQE-F

5' GCGTATCACGAGGCCCTTTC 3'

LacI

5' GCGAATTCAACATGGAACCAGTAACGTTATA 3'

EcoRI

EXAMPLE 5

Expression of the three *Eucalyptus* MADS-box genes in a range of tissues within two *Eucalyptus* species

Northern blots were performed on a range of tissues in order to determine the tissue specificity of the expression of the three EGM MADS-box genes. Tissue used for isolation of RNA from roots, seedlings, stems, shoots, leaves and mature flowers was obtained from Eucalyptus grandis plants. Tissue for isolation of RNA from floral tissues including receptacles, petals, stamens, carpels, and styles was collected from *E. globulus* flowers. This species was used because it produces very large flowers, making collection of sufficient amounts of RNA much easier. Ten micrograms of total

RNA was used in the blots which included the different floral tissues and 20 micrograms was used in blots of *E. grandis* RNA which included the vegetative tissue. Blots were probed with the three EGM genes which had been digested with the appropriate restriction enzymes to remove the MADS-box.

The northern blots indicated that all three EGM genes are expressed at a high level in eucalypt flowers. When northern blots are exposed to film for long periods, weak expression of the EGM2 and EGM1 genes was detected in floral tissue. The EGM3 gene was specifically expressed in vegetative tissue. Within the flower, the EGM2 gene was observed to be expressed in stamens and petals. The EGM3 gene is expressed in receptacles, petals, stamens, carpels and styles.

EXAMPLE 6

DIRECTED DEVELOPMENT STRATEGY

This strategy involves the expression of an antisense version of a gene critical to the development of reproductive structures so that vegetative development occurs instead.

The MADS-box region of PrMADS1 gene was fused in the frame to the amino end of GUS reporter gene's coding region in sense and antisense orientations (Fig. 47). Constitutive expression of these genes will lead to high level of accumulation of the corresponding products in cytoplasm and nucleus. Accumulation of the fusion proteins containing sense orientation of the MADS-box region could lead to the inhibition of downstream transcription processes through competitively binding to specific trans-acting elements in the promoter regions.

Antisense orientation could block expression of MADS-box genes through RNA-RNA interactions between fusion antisense MADS-GUS mRNA and target MADS-box mRNAs in floral meristem.

Constructs were introduced into the binary vector Binl9 and transformed into *AGL1* Agrobacterium strain. *Arabidopsis thaliana* plants were transformed using a root transformation procedure and transformants were selected on medium containing kanamycin antibiotic. Only antisense constructs were analysed for floral inhibition.

The 25-30% of transformants with antisense MADS-box genes (lines FB13L) did not induce any inflorescence even 45 days after transferring to the soil. These plants look very bushy with much larger leaves than controls (Fig. 46). After 45 days control plants had already passed through all vegetative stages and finally gave seeds. Usually, in *Arabidopsis* plants floral buds have already appeared when 8-10 rosette leaves are appearing.

In FB13L plants there was no detectable floral induction even after 25-35 leaves. Leaves of FB13L line plants were stained with X-gluc (histological GUS assay). Most of the plants have shown the blue color of the leaves which indicates a high level of accumulation of GUS proteins.

EXAMPLE 7

Transformation of *Eucalyptus*

A number of abbreviations are used in the following text. These are in common use in the field of plant tissue culture.

BA: benzyladenine also known as 6-benzylaminopurine.

IBA: indole-3-butyric acid.

NAA: 1-naphthaleneacetic acid

TDZ: thidiazuron also known as 1-phenyl-3-[1,2,3-thiadiazol-5-yl] urea.

The following is a detailed description of the preferred steps for producing transgenic *Eucalyptus* from shoot and seedling

explant material.

Shoot explants

1. Subculture shoots monthly on solid KG medium containing 0.2mM BA. Keep in low light (16 hour photoperiod, 100-350 Lux or 1-8 $mmolm^{-2}s^{-1}$ PAR) and 22.5°C.

2. Use whole shoots 3-4 weeks after subculture.

3. Remove lower leaves leaving the top 4-6 leaves.

4. Wound leaves 5 to 6 times with a needle (e.g. 25G) and place whole shoots in an *Agrobacterium* suspension (approximately $1x10^8$ cfu ml^{-1}) containing 10-100mM acetosyringone for 10 minutes to 2 hours. An *Agrobacterium* suspension with an optical density of 1.0 at 600nm diluted 1/20 is approximately $1X10^8$ cfu mL^{-1}. Best results when wound near base of leaf. Alternatively, instead of leaving the wounded shoots in an *Agrobacterium* suspension for 1 hour, the shoots can be vacuum-infiltrated with the *Agrobacterium* for 10-30 mins at 40-100 Kpa. The shoots do not need to be wounded for the vacuum infiltration procedure. However, callus formation is greater when wounding is effected with a needle rather than by vacuum-infiltration.

5. Blot shoots between sterile filter papers and insert shoots vertically into KG medium containing 0.2mM BA. Co-culture for 2 days in the dark.

6. Transfer shoots (still upright) to KG medium containing 0.2mM BA and 200 mgL^{-1} cefotaxime. Keep in low light for 5 days (16 hour photoperiod, 100-350 Lux or 1-8 $mmolm^{-2}s^{-1}$ PAR).

7. Excise 4-6 upper leaves and place them, adaxial face up, on solid callus induction medium G22 containing 2mM BA, 2.5mM NAA, 200 mgL^{-1} cefotaxime and 5-10 mgL^{-1} geneticin. Incubate for 2 weeks in the dark.

8. Transfer explants to G22 medium containing 2mM BA, 2.5mM NAA, 1.0mM TDZ, 200 mgL^{-1} cefotaxime and 15 $mg.L^{-1}$ geneticin.

Subculture every 2 weeks in the dark. Brown phenolic compounds are produced when incubated in the light.

9. After 6 weeks transfer explants to shoot induction medium GBA (i.e. G22 containing 5mM BA and 0.5mM NAA) containing 200 mgL^{-1} cefotaxime and 15 mgL^{-1} geneticin. Leave in dark for 5-6 days then move into light (16 hour photoperiod, 100-350 Lux or 1-8 $mmolm^{-2}s^{-1}$ PAR). Subculture every 2 weeks.

10. After 8-10 weeks on GBA with 200 mgL^{-1} cefotaxime and 15 mgL^{-1} geneticin transfer pieces of callus with buds and callus formed on the original explants to GBA with 200 mgL^{-1} cefotaxime and 30 mgL^{-1} geneticin. Subculture every 2 weeks.

11. After 4-6 weeks on this medium place regenerated shoots in liquid KG medium containing 0.01 mM BA, 50 mgL^{-1} cefotaxime and 5 mgL^{-1} geneticin for 2 weeks then transfer to medium with higher geneticin (10 mgL^{-1}) for 2-4 weeks. The liquid cultures are shaken at 100-120 rpm with 8 ml of liquid in a 70 ml container.

12. Transfer surviving shoots and callus to solid medium (KG containing 200 mgL^{-1} cefotaxime and 10 mgL^{-1} geneticin but with no hormones) and higher light intensity (16 hour photoperiod, 450 Lux or 10 $mmolm^{-2}s^{-1}$ PAR). Subculture every 2 weeks.

13. Assay putative transformed shoots for marker gene(s) activity.

14. Regenerate plants from the confirmed positive shoot material. Induce rooting by transferring shoots onto KG containing 10 mgL^{-1} geneticin but with no hormones. However, if there is no rooting after three weeks then move to the same medium containing IBA 0.2 mgL^{-1} (9.8 mM).

Seedling explants

1. Disinfect seeds and germinate on KG containing 0.2 mM BA.

2. Using 10-12 day old seedlings, remove roots and place them in an overnight-grown *Agrobacterium* suspension (approximately

$1x10^8$ cfu ml^{-1}) containing 50mM acetosyringone. An *Agrobacterium* suspension with an optical density of 1.0 at 600nm diluted 1/20 is approximately $1X10^8$ cfu mL^{-1}. Wound the cotyledons and hypocotyl by gently stabbing with a 30 gauge syringe needle under a dissecting microscope. Incubate for 1 hour then remove the seedlings from the suspension and blot them between sterile filter papers to remove excess liquid. Instead of leaving the wounded cotyledons in an *Agrobacterium* suspension for 1 hour, the cotyledons can be vacuum-infiltrated with the *Agrobacterium* for 20 mins at 95 KPa (28mm Hg). The cotyledons do not need to be wounded for the vacuum infiltration procedure. However, the hypocotyls require wounding even when using vacuum infiltration.

3. Co-cultivate on KG medium (containing 0.2 mM BA) for 2 days in the dark making sure that the seedlings are standing upright in the medium.

4. Transfer the seedlings (still upright) to KG medium (containing 0.2mM BA) and containing 200 mgL^{-1} cefotaxime. Continue incubation in low light (16 hour photoperiod, 100-350 Lux or 1-8 $mmolm^{-2}s^{-1}$ PAR) for 5 days.

5. Excise the hypocotyls and cotyledons. Transfer the hypocotyls to callus induction medium G22 containing 0.5mM BA, 1.0mM NAA, 1.0mM TDZ, 200 mgL^{-1} cefotaxime and 10 mgL^{-1} geneticin. Transfer the cotyledons to G22 medium containing 1.0mM BA, 1.0mM NAA, 0.3mM TDZ, 200 mgL^{-1} cefotaxime and 10 mgL^{-1} geneticin. Continue incubation in the dark for 2 weeks.

6. Transfer explants to the same medium containing 15 $mg.L^{-1}$ geneticin and 200 $mg.L^{-1}$ cefotaxime. Continue dark incubation. Subculture every two weeks until about 6 weeks have elapsed.

7. After 6 weeks, transfer to shoot induction medium GBA (i.e. G22 containing 5mM BA and 0.5mM NAA) containing 15 mgL^{-1} geneticin. Leave the cultures in the dark for 5-7 days then

transfer them to the light (16 hour photoperiod, 100-350 Lux or 1-8 $mmolm^{-2}s^{-1}$ PAR).

8. After about 10 weeks on the GBA with 15 mgL^{-1} geneticin and 200 mgL^{-1} cefotaxime, a number of explants will have produced shoots. These shoots are excised and transferred to KG medium (containing 0.2mM BA) with 30 mgL^{-1} geneticin and 200 mgL^{-1} cefotaxime. Meanwhile, the callus that has also formed on the original explants is subcultured back to GBA with 15 mgL^{-1} geneticin and 200 mgL^{-1} cefotaxime and left for a further month, by which time more shoots may develop which can then be transferred to KG medium (containing 0.2mM BA) with 30 mgL^{-1} geneticin and 200 mgL^{-1} cefotaxime.

9. All shoots and callus are now transferred to fresh KG medium (containing 0.2mM BA) with 30 mgL^{-1} geneticin and 200 mgL^{-1} cefotaxime for another month or so.

10. After 4-6 weeks on this medium place regenerated shoots in liquid KG medium containing 0.01 mM BA, 50 mgL^{-1} cefotaxime and 5 mgL^{-1} geneticin for 2 weeks then transfer to medium with higher geneticin (10 mgL^{-1}) for 2-4 weeks.

11. Transfer surviving shoots and callus to solid medium (KG containing 200 mgL^{-1} cefotaxime and 10 mgL^{-1} geneticin but with no hormones) and higher light intensity (16 hour photoperiod, 450 Lux or 10 $mmolm^{-2}s^{-1}$ PAR). Subculture every 2 weeks.

12. Assay putative transformed shoots for marker gene(s) activity.

13. Regenerate plants from the confirmed positive shoot material. Induce rooting by transferring shoots onto KG containing 10 mgL^{-1} geneticin but with no hormones. However, if there is no rooting after three weeks then move to the same medium containing IBA 0.2 mgL^{-1} (9.8 mM).

Seedlings of 12 days old or younger are best for transformation but seedlings up to 20 days old can be used for regeneration.

Shoot regeneration frequencies for non-transformed cotyledon and hypocotyl explants without selection are approximately 80%.

Experience has shown that removal of cefotaxime from the medium, even after 3 months, will result in rapid overgrowth by *Agrobacterium*. The concentration of cefotaxime varies between liquid and solid media.

Shoot material grew faster in liquid medium and formed more shoots when compared to shoots grown on solid medium. This liquid selection step has the advantages of reducing false positives by increasing selection pressure, reducing residual *Agrobacterium* and increasing the amount of shoot tissue compared to solid grown cultures.

The regenerated rooted transgenic plants are then moved to a soil based medium and grown into trees of a size and form suitable for planting. Clones can be micropropagated by tissue culture propagation techniques and grown into trees of a size and form suitable for planting.

The media used in this study were G22, GBA and KG as described by Laine and David (1994). However, various basal media, including MS, B5 and P24, have been tested and found to support shoot regeneration. The plant growth regulator regimes were generally different from those of Laine and David (1994), which were in the combination of 1-3 mM BA, 0.05-2 mM TDZ and 0.5-2.5 mM NAA for callus induction from leaves; 1-3 mM BA, 0.05-1 mM TDZ and 0.5-2.5 mM NAA for cotyledons; and 1-3 mM BA, 0.05-2 mM TDZ and 0.5-2.5 mM NAA for hypocotyls. The differentiation medium was generally a

GBA medium, which was a G22 but supplemented with 2.5 - 5 mM BA and 0.5 mM NAA (Laine and David, 1994). A KG medium containing 0.2 mM BA is generally used as subculture medium for clone materials. All media were solidified with 0.25% Gelrite or Phytagel. pH was adjusted to 5.7 - 5.8 using potassium hydroxide before autoclaving for 15 minutes at 121

C. A preferred method for the preparation of Agrobacterium inoculum is described below.

1. Streak out the Agrobacterium strain containing the construct onto plates with selection, eg YEP + rifampicin (50 mg/L) + kanamycin (100 mg/L) for LBA4404, EHA105 and AGL1 containing the pBin GUSINT construct.

2. Incubate plate at 28oC for 2 days.

3. Pick a single colony into YEP broth with selection and grow on shaker overnight at 28oC.

4. Use the overnight culture to inoculate (1% inoculum) fresh medium with selection and grow on shaker overnight at 28oC. This additional step will produce a more consistent inoculum for plant transformation.

5. Harvest Agrobacterium, wash and resuspend in tissue culture medium. Dilute to an appropriate concentration ready for transformation.

6. Streak out the resuspended Agrobacterium on to lactose yeast medium plates and incubate plate at 28oC for 2 days. Do a Benedict's test (Bernaerts and Deley, 1963) on the colonies to confirm they are Agrobacterium.

This strategy will give good thick cultures of Agrobacterium on the day of transformation. A culture grown overnight which was inoculated from a plate or glycerol stock will give variable results.

An AGL1 culture grown as d scribed above and diluted t an optical density (600nm) of 0.98 had a viable count of 2.37×10^9 cfu/ml.

It will of course be realised that while the above has been given by way of illustrative example of this invention, all such and other modifications and variations thereto as would be apparent to persons skilled in the art are deemed to fall within the broad scope and ambit of this invention as is herein set forth.

CLAIMS

1. A method of enhancing vegetative growth in a plant including the steps of:-

identifying a gene having a substantially tissue-specific promoter expressing during the development of both male and female plant reproductive structures;

constructing an expression cassette comprising a heterologous coding region capable of expressing a product which aborts said development under the expression control of said promoter;

transforming plant cells with said expression cassette, and

selecting and vegetatively propagating the transformants.

2. A method of enhancing vegetative growth according to Claim 1, wherein said gene is selected from genes expressing specifically in the development of both male and female reproductive structures.

3. A method of enhancing vegetative growth according to Claim 2, wherein said heterologous coding region expresses a lethal gene product selected from lethal translation products and antisense RNAs.

4. A method of enhancing vegetative growth according to Claim 1, wherein said promoter directs expression of said heterologous coding region substantially in said both male and female reproductive structures, and wherein the effect of leakage of said promoter in non target tissues is suppressed.

5. A method of enhancing vegetative growth according to Claim 4, wherein said heterologous coding region expresses a lethal gene product selected from lethal translation products and antisense RNAs.

6. A method of enhancing vegetative growth according to Claim 5, wherein said suppression is provided by including in said expression cassette a control gene cascade functioning to prevent expression of, or counter the expressed product of said heterologous coding region.

7. A method of enhancing vegetative growth according to Claim 6, wherein said control gene cascade comprises a control gene selected to express a gene product acting to prevent expression of or counter the expressed product of said heterologous coding region, said control gene expressing under the control of a constitutive promoter which is itself under the control of a repressor gene the expression of which is promoted by a second tissue specific promoter.

8. A method of enhancing vegetative growth according to Claim 7, wherein said heterologous region codes for the enzyme Barnase, said control gene expresses Barstar under the control of a constitutive promoter including a LacIq op site, and said repressor gene expresses LacIq under the expression control of the same or another promoter specific to the same tissue as said heterologous coding region.

9. A method of enhancing vegetative growth according to claim 1, wherein said heterologous coding region expresses

under the control of a tissue specific promoter derived from the MADS-box genes of *Pinus radiata*, *Arabidopsis thaliana*, or *Eucalyptus grandis*, or strong homologues thereof of other species.

10. A method of enhancing vegetative growth according to Claim 9, wherein said plant is *Pinus radiata*, and wherein said tissue specific promoter is selected from PrMADS2, PrMADS3, PrFL1 or PrCon1.

11. A method of enhancing vegetative growth according to Claim 9, wherein said plant is *Eucalyptus grandis*, and wherein said tissue specific promoter is derived from the EGM3 gene.

12. A method in accordance with Claim 1, wherein said tissue specific promoter is identified from genomic DNA using a 'promoter finder' strategy (Clontech).

13. A method in accordance with Claim 12, wherein said identified cDNAs are selected for the presence of a single gene expressed in both male and female buds.

14. A method in accordance with Claim 13, wherein said promoter or, preferably, a constitutive is fused with a heterologous coding region which codes for an expression product of which is the antisense version of said gene.

15. The plasmid pBR Barnase prom Barstar 1 yielding V1-promoters according to FIG 21.

16. The plasmid pBR 35SPromOpBarstar yielding V2 promoters according to FIG 22.

17. The plasmid EGM3 double bin according to FIG 33.

18. The plasmid EGM3 V1 sense according to FIG 34.

19. The plasmid V1 (Barnase-Barstar) according to FIG 35.

20. The plasmid pBR Barnase according to FIG 36.

21. The plasmid V2 (LacIqNLS-35S promoterOp-Barstar) according to FIG 37.

22. The plasmid p35Sop Barstar E-2 according to FIG 38.

23. The plasmid p35Sop Barstar according to FIG 39.

24. The plasmid pBRlac.

25. The plasmid pBRGUS 1.

26. The plasmid pBRGUS 2.

27. The plasmid Bin 19+EGM3 V1 sense.

28. The plasmid EGM3 V2 sense.

29. The plasmid pBRLacBH-.

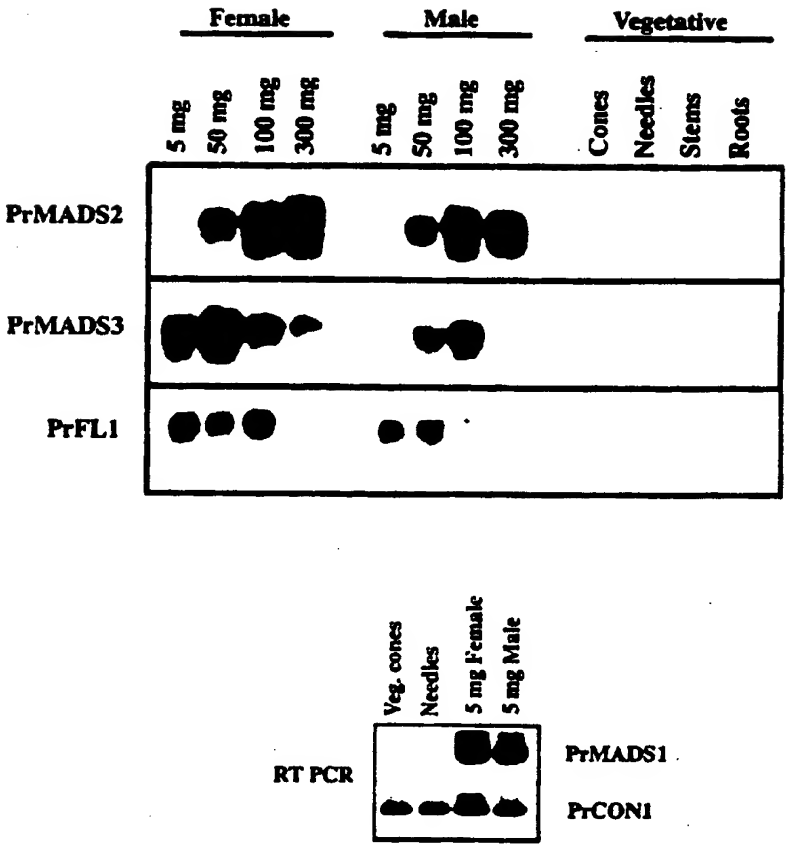

Female
Male
Vegetative
5 mg
50 mg
100 mg
300 mg
5 mg
50 mg
100 mg
300 mg
Cones
Needles
Stems
Roots
PrMADS2
PrMADS3
PrFL1
Veg. cones
Needles
5 mg Female
5 mg Male
RT PCR
PrMADS1
PrCON1

Figure 1

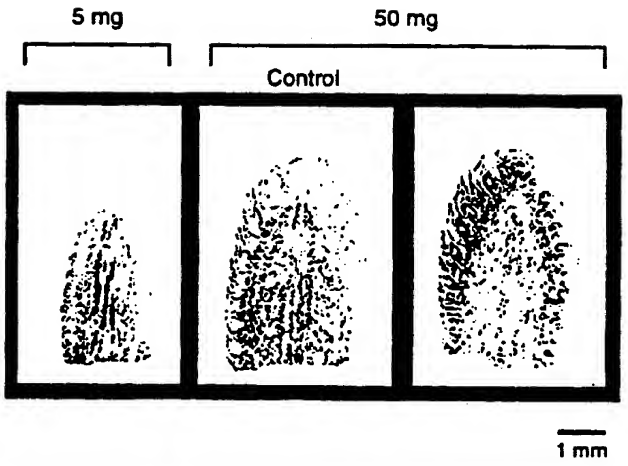


Figure 2

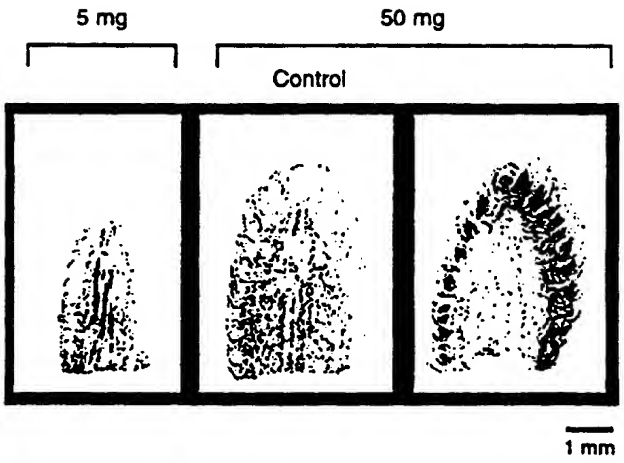


Figure 3

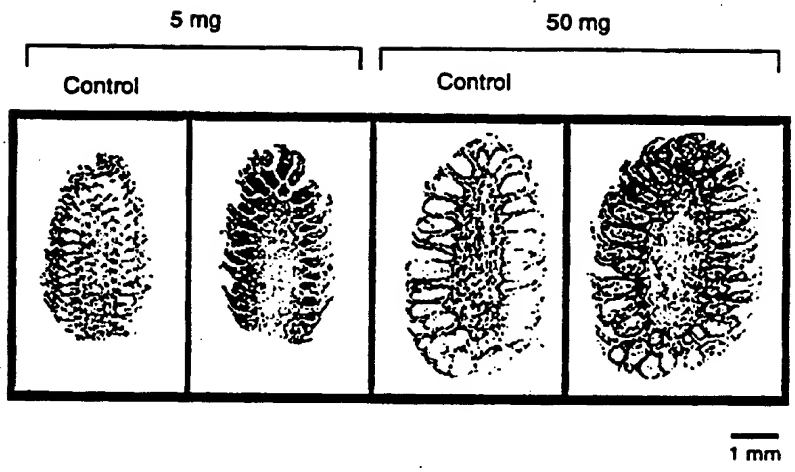


Figure 4

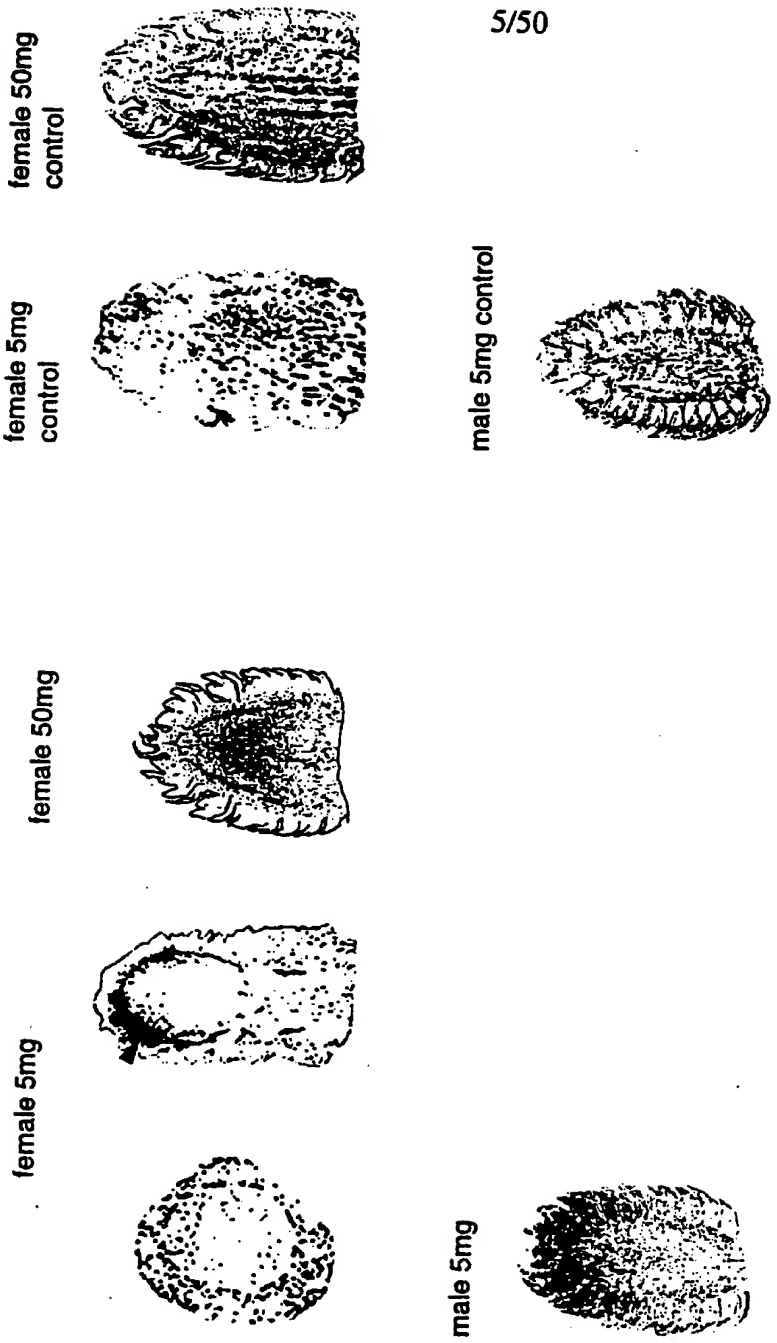
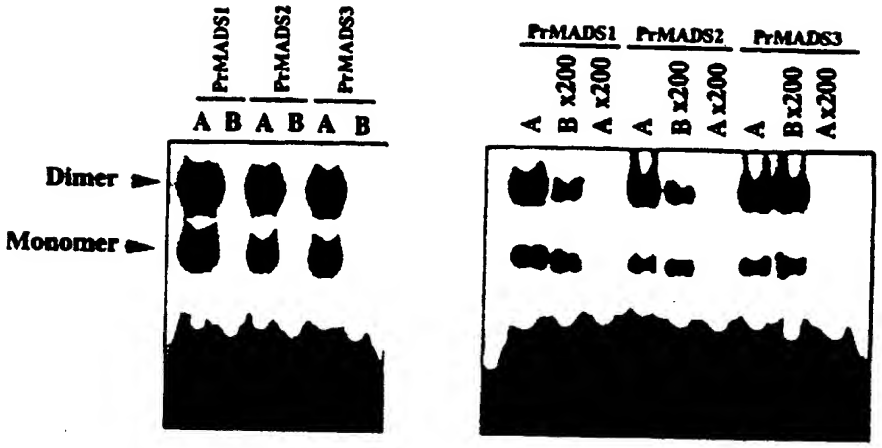

female 50mg control
female 5mg control
male 5mg control
female 50mg
female 5mg
male 5mg

Figure 5



AG consensus CArG-box
NTT(A/T)CC(A/T)NNGG(-G)(A/t)2N

A: TTACCAAAAAAGGAAA

B: TTAGGAAAAAACCAAA

Figure 6

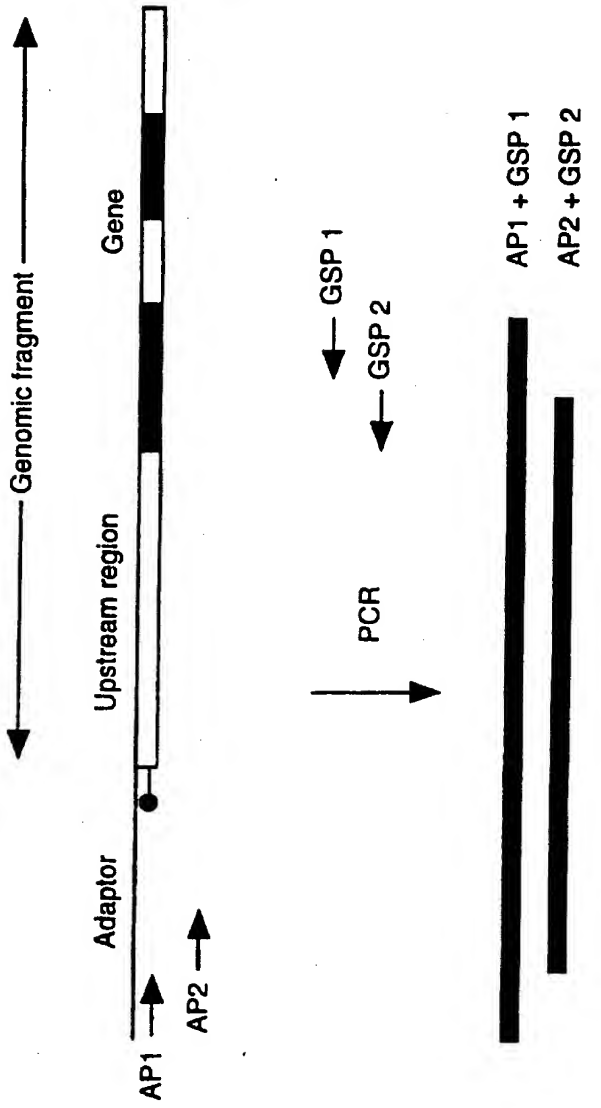

Genomic fragment
Gene
Upstream region
Adaptor
AP1
AP2
GSP 1
GSP 2
PCR
AP1 + GSP 1
AP2 + GSP 2

Figure 7

TGAGAAAGAGAGACAGAGATATGGGAAGAGGGAAAGTAGAGCTGAAGAGGATAGAGAA
CAAAATCAACAGGCAAGTAACATTTGCGAAGAGAAGAAATGGGCTTCTCAAGAAAGCTT
ATGAGCTCTCTGTTCTCTGTGATGCTGAGGTTGCGCTCATCATTTTCTCCAACCGTGGCAA
GCTCTATGAATTCTGCAGCAGTTCTAGCATGATGAAAACAATTGAGAAGTACCAGAAGTG
CAGCTATGGTTCACTTGAGAcCAACTGCTCCATCAATGAGATGCAGAACAGCTACCAGGA
TTATTTGGAGCTAAAAGCAAGAGTGGAGGTCCTCCAACGATCTCAGAGAAACCTCCTTGG
GGAAGAGTTGGGTCCCCTAAACTCGAAGGAGCTGGAGCAACTTGAGCACCAGTTGGAG
AATTCTCTGAAGCAAATTCGGTCTGCAAAGACCCAATTCATGTTTGATCAACTGGCTCATC
TTCAGCACAAGGAACAAATGCTGGTTGAAGCTAACAGAGAATTAAGGAAGAAGTTGGAA
GAGAGCAATACAAGAATCCCTCTCCGCCTTGGATGGGAAGCTGAGGATCACAATAACAT
TTCATACAGGCGCCTTCCCACGCAGTCGCAAGGATTGATCTTCCAGCCCTTAGGCGGCTA
CCCGAACATGCAGATCGGGTACAATCCTGCAGGCTCGAATGAATTGAATGTTTCGCCTGC
CGACCAACATCCCAacGGATTCATTCCCGGATGGATGCTCTGAATCGTTCCGCAAGTGAA
CTGCTTGCTGGAAGTTCCATATCAAGTACATTTTCCGGTTTTTGCTATGATATATGACTCTT
CTTCTTCTGGATGACCTATACGAAGATCCATCATTCGTGGATATTGTCCATGGACGTACCC
TAAAAGGAAGGACGGTATGAATCCAATCTAGCTTACTATTTTGTATAAGAATAAAGATCT
GTGCTGCTGATATTTGGAATTCATCTATGTTATTTAATGTATGGAATCC

Figure 8

MGRGKVELKRIENKINRQVTFAKRRNGLLKKAYELSVLCDAEVALIIFSNRGKLYEFCSSSS
MMKTIEKYQKCSYGSLETNCSINEMQNSYQDYLELKARVEVLQRSQRNLLGEELGPLNSKEL
EQLEHQLENSLKQIRSAKTQFMFDQLAHLQHKEQMLVEANRELRKKLEESNTRIPLRLGWEA
EDHNNISYRRLPTQSQGLIFQPLGGYPNMQIGYNPAGSNELNVSPADQHPNGFIPGWML

Figure 9

GAATTCGCGGCCGCTAGAAGAATAAGGAAGAGGCCGAGAGAGGGAGCATTATCGTCGA
GCAGGAGGGGGAAAGAAAGAAAGAAAGAAGGAAAGAATGGGGCGAGGGCGCGTCGAG
CTGAAGCGGATCGAGAATAAGATTAACCGCCAGGTCACGTTTTCGAAACGCCGGAATGG
TCTGCTGAAAAAGGCGTATGAACTTtCAGTGTTATGTGATGCAGAGGTAGCACTGATaATA
TTCTCAAGCAGAGGAAAACTCTATGAGTTCGGAAGCGCCGGGATGCTCAAGACTCTGGA
GCGATATCAAAAATGTTCATACGTATTGCAAGACGCGACTGTATCGgACCGGgAGGCGCA
GAATTGGCATCAAGAGGTtgGcAAATTAAAAGCCAGAGTTGAACTTTTACAACGATCACAA
AGGCACTTATTAGGTGAAGACCTGGGCCCCTTGAGTaTTAAGGAGCTGCAACAACTGGAA
CGTCAACTTGAGGTTGCACTGACACATGTTAGGTCAAGAAAGACTCAAGTCATGTTGGAA
ATGATGGATGAACTACGCAGAAAGGAGCGAATTTTACAAGAAGTAAACAAATCTCTGCG
CAAGAAGTTGCAGGAGGCCGAGGGACAGGCATTCAATGCCATGCAACCTCCCCCTCATG
CATGGGATTCGCATGCAGTAGCAAATAATGCATATGCTATGCAACATCCATCAAATGCTG
TGGATTGTGAGCCCACATTGCAGATTGGATACCAaTATGCCCCTCCTGAGTCAAGCATGC
CTAGGCATGAACAAGCACAAAACAATTACATGCAAGGCTGGATGGTATAATGTACCACCA
ATTTCTAATGACAAGCAGAAGTATTAATATTTATAAATATTAAAATGTCTGTATATATAGCT
ATGAATTTACAGTTGTATTGTACTTCCATTTTAATTCATGTTTTTCAGTAGTTTGATTGTAG
TAACTTAACATTTTATTTTTCCCAAACCCTAACAACTTTGACTTTTAAAAAGTCACAATTTTA
TGCAACAATATTTGTGTTTATAtATATAATTGCTTTTCCTGTTTAAAAAA

Figure 10

MGRGRVELKRIENKINRQVTFSKRRNGLLKKAYELSVLCDAEVALIIFSSRGKLYEFGSAGM
LKTLERYQKCSYVLQDATVSDREAQNWHQEVGKLKARVELLQRSQRHLLGEDLGPLSIKEL
QQLERQLEVALTHVRSRKTQVMLEMMDELRRKERILQEVNKSLRKKLQEAEGQAFNAMQPP
PHAWDSHAVANNAYAMQHPSNAVDCEPTLQIGYQYAPPESSMPRHEQAQNNYMQGWMV

Figure 11

CTCCCGGCCGCCATGGCCGCGGGATACTATAGGGCACGCGTGGTCGACGGCCCGGGCT
GGTAAAAGACGTCCCTCCGATGAATTCGACAGTGGAGTCCACAAAGAAAGATGCGTACG
CTGAATGCCACGTGTCTATAGTTTGCCCACACCTTTCAAACATACTCTGTACGCAGATCG
GCGGACCTGAAAATTTTCTTCGTATAAATCGTCTGTGTTAAGGATATTTGACCATCGGTCC
GAAAGATGCGACTGTCCGACTGCATCTCGTCTCTGGTAATGTTTTTTTTCAAATTTATAAA
GATTGTTTTGCATTATTATTATTTATCATGTTGCGTAAgATCTGGTGAGCAGTATTATTTGT
CACTTTGTGTGGAATCCCTTATGAAAATATATATTTATtCCAGAAAAgTATTTTAGCTTGTCA
AATTGTGTGGAATCTGGTATAAGTACCTGTATTTATCAGACGTATTATTTGTTAAATTGTG
TGGAATCTGGTATAAATTTATATACTTCGTGAGGAAATGTATTTATTAGCAGTATTACTGG
TCAAACAGAGTGGAATCCTGTATAAATTTATCATATTTAGGAACAAATGTATTTATCAGCG
TTATTATCTGTCAAAATGTGTGGAGACGCGATGACTTTAATTTTTTGATAATAGGCGGAGC
GTATAGGTACGCTGTAGTATTGTAGACTGGCATGTAGAGTACAAAGTTTAGATATTAACC
TAGATTATTTTTGCTATTCGCAGGCTATCGCGTACTCATGGTGAAATGCGTACAGTTTCAA
AGTAAATGGTTGATGTTGAGTGCGGTGGCGGGGGCAGACACACAAATAGGAATCGGAgA
gCGAGAGTACTGCAGCAATTGTTGTTTAcTTTTTGAGCAAGACGAGGATCAAAGAAGAAT
AAGGAAGAGGCCGAGAGAGGGAGcATTATcGTCGAGCAGGAGGGGGAAAGAAAGAAAG
AAAGAAGGAAAGAATGGGGCGAGGGCGCGTCGAGCTGAAGCGGATCGAGAATAAGATT
AACCGTCAGGTCAcGTTTTCGAAACGCCGGAATGGTcTGCTGAAAAAGGCG

Figure 12

ATGGGGCGGGGTCGGGTTCAGCTGAGGCGAATAGAAAACAAAATAAATCGACAAGTCA
CGTTTTCGAAGCGCCGGAACGGACTGCTGAAgAAgGCGTACGAGCTATCAGTGCTGTGC
GATGCCGAAGTGGCGCTAATAATTTTCTCTACCAGAGGAAAGCTTTACGAGTTTGCCAGT
TCCAGCATGAACAAGACGTTGGAAAGATACGAAAAATGTTCATATGCAATGCAAGATACC
ACAGGCGTTTCGGACCGGGAAGCACAGAATTGGCACCAAGAAGTTACAAAGTTGAAGGg
TAAGgTTGAGCTCCTGCAGCGATCACAAAGGCATTTGTTGGGGGAAGATCTGGGTCCGTT
AAATGTTAAGGAgCTACAGCAGCTTGAACGTCAGCTGGAGgTTGCTCTGACACATCTTAGg
TCaAgGAAAACGCAGgTAaTGCTGGACCAgATTGAGgAaCTTCGCCAAAGGGAACGGTTGC
TACATGAAGTAAACAAGTCTCTGCAGAAAAAGCTTTCCGAAACAGAGGGAAGAGATGTAa
TAACTGGCATAgAGCAAACTTCTAATACTAATACTGGTACTAACGGTCCTTGGGATTCTTC
TATCACAAACACTGCgTATGCTCTCTCACACCCTCAACAAGATTCAAATTCAAGCCTCCAC
CATGTGGACTGTGAACCCACGCTACAGATTGGTTATCAGCCTGTGGCTCCTGAAAGCATC
GTCCCTCCTCATCAGCCGCCGCACAACCAAACGCCGAAcCAATACATGCAAGGATGGTG
GGTTTGATATTTAACATTTATCATTATCAGTTACTTCAATCACAACAAAAGCCCAAAGCGT
GGTAAATTACGAAATTAGAATTATATTATCATTAAAAAAAAAACCCTATTTTCATTGTATAgC
AGTAGGCTTGATTTACTGCTATGATAGCGGAGGTTTTATTGGGCAAACAAACCCTACTGG
TATATTAGACCTTCTTGTCGACAAAGTTTAATTGCATAAATCTTGTATGCTAATCTGGCCG
CTAAAAGAgCGATGGAAAAATAGTTGTCCCATTCACAACACATGATATGTTTAAATCCAAC
GTGTATGTGTCTGCAAAATATTATTATACACTACGGTTTATCAAAAAAAAAAAAAAAAAA

Figure 13

MGRGRVQLRRIENKINRQVTFSKRRNGLLKKAYELSVLCDEAVALIIFSTRGKLYEFASSSM
NKTLERYEKCSYAMQDTTGVSDREAQNWHQEVTKLKGKVELLQRSQRHLLGEDLGPLNVK
ELQQLERQLEVALTHLRSRKTQVMLDQIEELRQRERLLHEVNKSLQKKLSETEGRDVTTGIEQ
TSNTNTGTNGPWDSSITNTAYALSHPQQDSNSSLHHVDCEPTLQIGYQPVAPESIVPPHQPPH
NQTPNQYMQGWWV

Figure 14

GGCCCGGGCTGGTATTTGGTGTAAGTTTTAAAGGAAGCTGGAAGATTTCAAATCAGTCCT
AATCCTTTAAAAAcTCAGCTTTTGCAAACATGTAACAATCTATGAGTATTATTATGCGTTAT
GGATGTGGCATTTCCACTGCCTTTTAAGTACTTCGGTTTGTAAGTAAAACTAAAACAGAAA
ATTTCTCTTCTAGTATTCTTAAACCGAGTGGAGTTGAGCTGAAGTGAGAGGAGGTATATA
TATATATGTATATTTATTTAGAGTCCAAAGAATCGAGGCGAAAGGCATGTGATGGACGAA
AGCAACCTTAATCTCTCTGCAAATTGTCCCTCCAAATTCCAACTAAAATCAATCCGGACCC
GCATGAGCATCGATCGAATCATTTGGGTCATGCATCCTATGCATGCTCATCGATAACGAT
AATGTATGGGCATGTGGCGCTCATCAATGATGATAGATGGGCATGGGGCCCCACATGAT
GTGGACAGTGGCAACAATAGTACACTAGTTGTAAATGGGTAACCTGTAGTTGGCTTGGTT
TACTTTATATGGAATGAAAGAGGTACGAAACAAAGTATATCAAACGTTGACTCAATGAGT
ACGCCGATCGACGCGTGTTTTTAATTTTTTAATGTTCCAAGGCCGGGCAGGCCCACTTCA
ATCAACGCAATGCCACGCCAAAATTCACCGATATTTATGTCATGTTTTAACTAATAAATTA
CCCAGCGGAAAACTGGTCGCAGCGTAAAGCGTTCATGGGTGCCGGGCGGGTAACTCTT
GAAAAATGTATACAAATTTATTTATATGAAAAAATATCATGCTGATGATATACGCACGTTC
TCTCTcTAGGTTTCTTTTTCTTGCTTTCTTTCTGGTCCACATAAACTACcTACTCTTATAATGT
GCGTGTCATTGAGGTAGATTAGATTCGATTCCCTGACCCTGGGAGGAGGAAGAAGAAGA
AGAACAGCAGGAGGAAGCGAAAATTTATTAATAGTAACCAGAGAATAGCAGCGGGTGAA
GAAGCAGAGGGATCTTGCAATGGGGCGGGGTCGGGTTCAGCTGAGGCGAATAGAAAAC
AAAATAAATCGACAAGTCACGTTTTCGAAGCGCCGGAACGGACTGCTG

Figure 15

GAAATTCGGCACGAGGGTAGAGAGATCCCTGGGCCTAGAAGTGTTTTCGGAGGAGCGC
ACTGCATTCTACTTCGGAAAAAAATATGGATGCAGAGCACTTTCCTGTAGGTTTCTTTAGG
TGGGATCAGAGACCAGCACCAGTTGTAGCGGCAGCAGCAGCACCAACAACAACTGTCTT
TAACAAGGACCATGGACGACCGTTGGAAgTCaTTCTtCCCATGAATGGGAGAAAGGATTTG
AAGTCCCTTGAAGATCTGTTTAAAGAGTATGGAGTTCGATACGTAACTCTTGCCAAGATG
ACCGAGATGGGCTTCACTGCCAACACCCTTGTCaATATGACAGAGGAAGAGATTGAAgAT
TTGATGAAGACCcTGGTAgAACTCTATCATATGGATCTTCTTATAgGGGAGAgATATGGAaT
TAAATCTGCCATAAgAgCAgAgAAgAAAAgGTTGCAGGATAgCTTGGAGATGCAAAgGTTGG
AAATCTTGTCTGAGGCAGAGAGAAAGAGGATATTACATGATGATCAGAATACTTTTGCAG
CTGCTATGGCATCCGAAGGAACATCTAAGGAACTGAGAGCAAATGACCCACTGATTTTCC
CAGAAAGCACAAGTGCAGATCATGCCCCAATGAATATAGCCAGCTGCAAAGACAGTACT
CTCATTCTCCAGAACAGTAACCAGGCACAGTTTTGTGGCTCGGGATTGATTGGAGTGCCT
GAGCACAGCAGTGAGAGCGATGAAAGGAAAGCTGATACGAATAAGCAGAAAAGGAGGC
GGTCCAAGGAGCCTGGAGAGGATGGGGAGGACAgGCCTAgAgAGCATCCTTTCATTGTC
ACGGAACCAgGAgAACTGgCAAGAGGGAAGAAAAaTGgTcTGGATtATcTCTTtgAtcTcTaTGA
GCAGTGtGGGAAATTTTTATtaGAAGTACAAaGGATTGcTAAGGAAAaGGGAGAAAaATGCC
CAACAaAGGTtACAAATCAaGtGTTCCGTCATGCCAAGCACAATGGTGCTGTctACATAAACA
AACcTAAAAtGCGACATTATGTTCATTgCTATGcTcTgCaTTGCttGGACAGTGAGCAATCCaaTc
ACcTCAGAAGaCTATACAaGGAGAGGGGAGtAAATGTTGGGGCCTGGCGCCAGGCcTGTT
AcTATCCCCTGGTAgCCaTAGCCAGAGAGAATAATTGGGATATTGAGGGCATTTTTAATAG
GAACGAAaAGCTTAAGATttGGTATGTTCCCACAAAAcTTAGACAACTGTGTCATATGGAGA
GAAGCAAAGAGTGTCAATAGTTTCATTGAGATTAATGTGTGTAATTTAACTTAGGCAGCT
GTGTCACATAGAGAGAAGCAAAAAGTGCCAATAGTTTCATTGAGATTAATGTGTGTAATT
TGAGCTGCTCAGCTTCAAGTGTAGCTCCGTGATTATTGGGCATTTGTGTTCTCATtGTGAC
CTTGCATATCAGATTTGATATGCATTGTGTCATGCCGTTcTTTCAAACATTGTATATGTATT
GGCAACTGGGTATGGATcTGCTCCTTTCTCCGTCACTAAACATGCGGAAACCTTTGTTTCT
CCTTGTGATAAATTTCAGTACATTACTGTTCATAGTTATTTTCTTTCAAAAAAAAAAAAAAA
AAA

MDAEHFPVGFFRWDQRPAPVVAAAAAPTTTVFNKDHGRPLEVILPMNGRKDLKSLEDLFKE
YGVRYVTLAKMTEMGFTANTLVNMTEEEIEDLMKTLVELYHMDLLIGERYGIKSAIRAEKKR
LQDSLEMQRLEILSEAERKRILHDDQNTFAAAMASEGTSKELRANDPLIFPESTSADHAPMNI
ASCKDSTLILQNSNQAQFCGSGLIGVPEHSSESDERKADTNKQKRRRSKEPGEDGEDRPREH
PFIVTEPGELARGKKNGLDYLFDLYEQCGKFLLEVQRIAKEKGEKCPTKVTNQVFRHAKHN
GAVYINKPKMRHYVHCYALHCLDSEQSNHLRRLYKERGVNVGAWRQACYYPLVAIARENN
WDIEGIFNRNEKLKIWYVPTKLRQLCHMERSKECQ

Figure 17

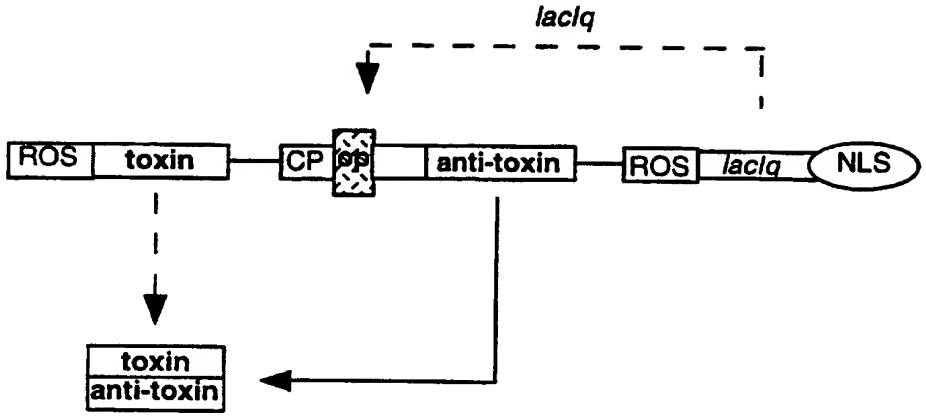
GCCGCACTAGTGATTCGTCCATGGTCCTTGTTAAAGACAGTTGTTGTTGGTGCTGCTGCT
GCCGCTACAACTGGTGCTGGTCTCTGATCCCACCTAAAGAAACCTACAGGAAAGTGCTCT
GCATCCATATTTTTTTCCGAAGTAGAATGCAGTGCGCTCCTACACCAAACAAAAACCACTC
ATAGAATTAGCACATGGGTGATAAATTCTGAgATGCTTTTCATTGATTACCGATTGAACTG
CAAgATTTGAAGTTGATGGGTTACAAATTCCTCCAgATATTTAACCAgACCAAgTGGGTTCC
AAGCGGTACTTGAAgAAgCAgAATgTGTACCTCCGAAAACACTTCTAGGCCCAGGGACCTC
TCTACCATCCAGTGACTTTACATTAgAAAATGTAAGGCTGCTCCCATATTTAAGGCTGTCTt
GTGAgTTTAATGCTtCTTAATCTAGTTTACTGGAACACCATTTTCTTCTAATCACATCaCAGT
AgTAgCTATCATCCAAACTTATCCAAGACAAAATATCTGAAACCATTCCTGAACTAATGATT
TGACTATACTTTGAGTTAGCGTTGTACTCAAATTATTCATTGCGGGGCCATGTTTGCAGGT
TAAACAAAAATCTCCACAGTCTGATTGCTATGTTTTATCAAATGATTTCTCAAATAATAATT
CAGTGTACCCATTTGTATTTTTACTTTAATACTTTTCATTAATGTCCTCGAGAGCAATGGCT
CTAGGGTCAAAGGAACCTTATCCCAGTACCGTACGCTGTAAATACTGTAATGTTGCTTTTA
TGGTGTCCTATAGATTACAGCGAGACTTGCTAGTCATGTGACTTTTAAAAATTTCATGTCA
GGCACTTGAAAATTGTTTCAAGTACAATGCAATTTTCCCATGGACTACTGGAACTGCCTG
CTAAAACTGTCTTTTCATGGTCTAATGAGAAGTATATGACCAGGCCTCTTCTCCCTCCATT
TTAAGAAAAACAAATTAAATTATTTAAAAAATAAAATTACaATAATAATCTTGACAGATTGA
TTTTGAGTTGAATGTTAGAATAGATGGAGTGCTGCCTAAGATTTGTTGGCCAAGAAATCC
GCAGCTTATTCCAGAAGTTCACTCATTACAATGTTTTATCTAAGAAATCCTCAACTTATTCC
AAAATGTAACTTATTACAATACCAGCCCGGGCCGTCGACCACGCGTGCCCTATAGTATCC
CGCGGCCATGGCGGCCGG

Figure 18

GGCACGAGCTCGTGCCGAATTCGGCACGAGCGAGTTTTTTTTTTTATGGTATTGGAGAAT
TTCGGCGGAGTTGGAGCACGGGAAGCcTCGTGCCGAATTCGGCAcgAGCTCAgAGGGATT
GGAGGGTGGAGAATGAgTATGCCAAAGCTCTGTGATGTTTGTCaGGTATCGAGCTCTGTA
ATATATTGCAGAGCTCATACTGCACAGCTTTGCTTAGTCTGTGATGCTAAAATTCATGGTG
GTAGCAAGgCTTCGTTGTGTCATGAAAGAgTTTGGGTTTGTGAAGTATGTGAGCAGGCCC
CAGCTGTGGTTACATGCAAGGCAGATGCAGCAGCTTTATGTGTAGCCTGTGATACTGATA
TTCATTCTGCCAATCCTCTTGCAAGTAGGCATGAAAGAGCACCTGTGATTCCATTTTATGA
GTGCCCTAATATGCCCACTAATAATACAGTTACACATGCCAATAATGATAACTTGGACTGC
AATGTTTTGCTGAATGAAGATgGTGGTGGGGATGaTCcTCTGAAACATGATTATGTTGATG
ATGACTATGGTGATTATGATGATGATGAGAATGATCAGAACAATTTGTTGAACAATCaGG
AAgATAATAATGATGCTGAAATATGTTGTGCAgAAgAGgCTGCAACAGCATCATGGTTGAT
TCCTGAGGCCAACAGAAATAATTTGACAATTATCAATGGAGGTAACTCAGAAGGAGAAG
ATAAGATGGTGAAGGATAAACTCAAGTTCAAGGCCTATATGCAGAGCATGGATTTTTTAC
AAGATGTGGATAATTACGCCGATCTGGAGTACTTGGGAACAACTACTATTACAACGCCAA
TAAACCCCACTGCCAATATGGGGGCAGATAGTATGGTTCCTGTTCACACTCCTGAAGTTA
TTGAGCATTCTTCTACAAAAGTTTCTATTGATACAGCTGGGTCAATGGATGTGGATGCAG
CATCCAAGTGCAATCACgTTTACAGAACTACATCTCTCAATCACtGTGTCTCTTCCTCCCCC
ATAGATGTtGGAATTGTACCTGACAGCAACATTACATCTGATATTTCAACACCTTACCATG
ACCCAAGAGGAGTATTCGAGATTCCTCCTCGGGTTGTTCATCCTGGAGGCCAAGGTGAG
GTCATGGGAAgAGAAgCAAGAGTTCTCAGATACAGAGAAAAAAGAAAGAACAgAAGGTTT
GAGAAgACAATACGATATGCTTCTAGAAAAGCCTATGCAGAGACTCGGCCCAGGATAAAA
GGCCGATTTGCCAAGAGAACAGAGGTAgAAGTGGAACAGATATACTCATcTTCTTTGCTTC
CTGATCAAGGATATGGAGTTGTTCCATCTTATTGAACCAGTCAATATAAATAATTAACTCT
TCTCTAGTATCTATCCTTGTTATTCTAATTGTAACTGCCATCACAATCATAATGCCTTATAA
CTTCAATTTATTCATGCCTATGAGATTGGGCTGGTATGTaAAACTCATGGCAGGTCTATTCa
CATTTGGGTTGTTCGCATACTCAAAGCCTCCTCCTTACTCATTCTGCCTTGCTCATTGATgT
GGgTCAGACTGCAGTAAGATGAAGTCGGTGTGACTCACACTTTGAAGAACAGCCATACAT
ACTATTAGTGTTTCAAGTCGATCGAAGTTTGTTTATTCAGCTTCCTGCAATCTTCTCTGCTC
ATGAATAAATCTTTTCCCCAGTCTCCTACTAAAAAAAAAAAAAAAAAA

Figure 19

A. non - flowering period
(in all tissues)



B. flowering period
(in reproductive tissue)

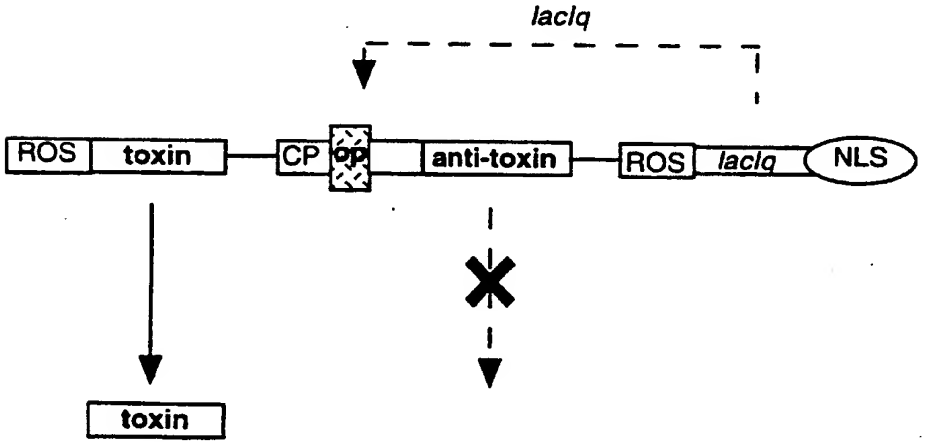


Figure 20

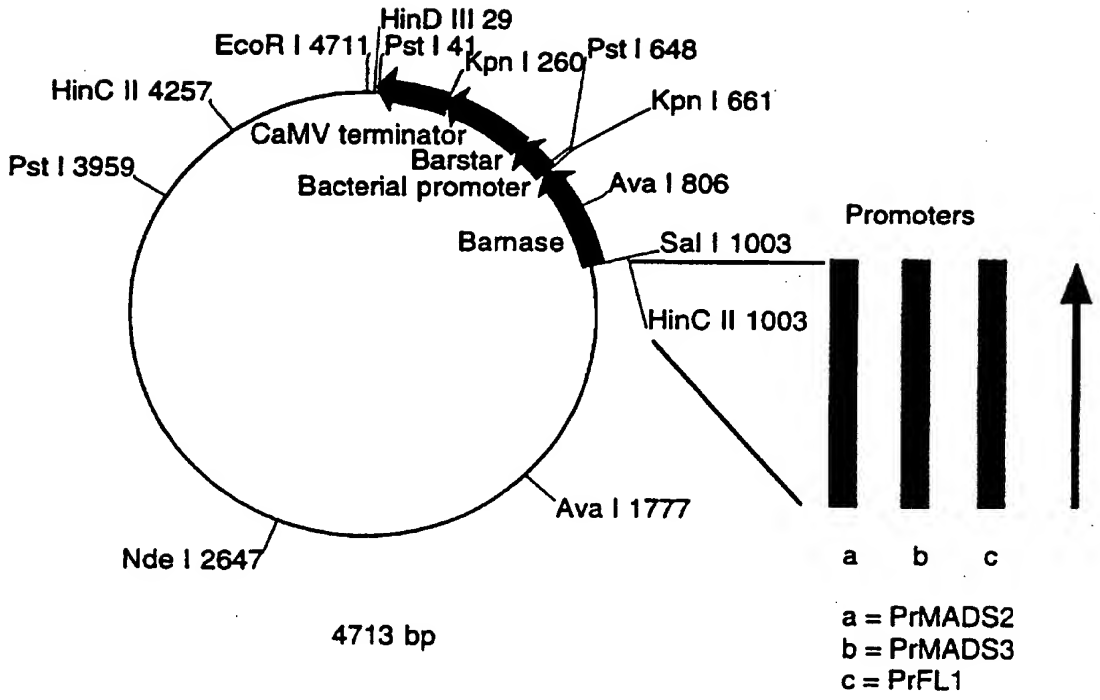

HinD III 29
EcoR I 4711
Pst I 41
Kpn I 260
Pst I 648
HinC II 4257
Kpn I 661
CaMV terminator
Barstar
Pst I 3959
Bacterial promoter
Ava I 806
Promoters
Barnase
Sal I 1003
HinC II 1003
Ava I 1777
Nde I 2647
a
b
c
4713 bp
a = PrMADS2
b = PrMADS3
c = PrFL1

Figure 21

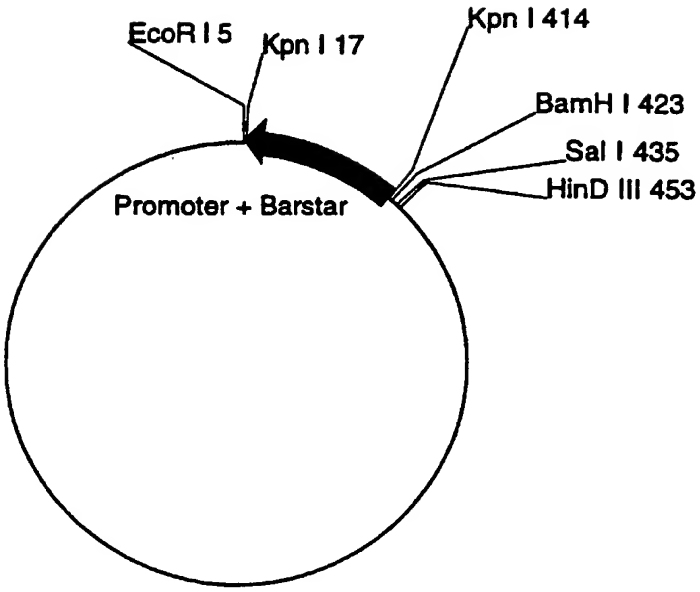

EcoR I 5
Kpn I 17
Kpn I 414
BamH I 423
Sal I 435
HinD III 453
Promoter + Barstar
3596 bp

Figure 21a

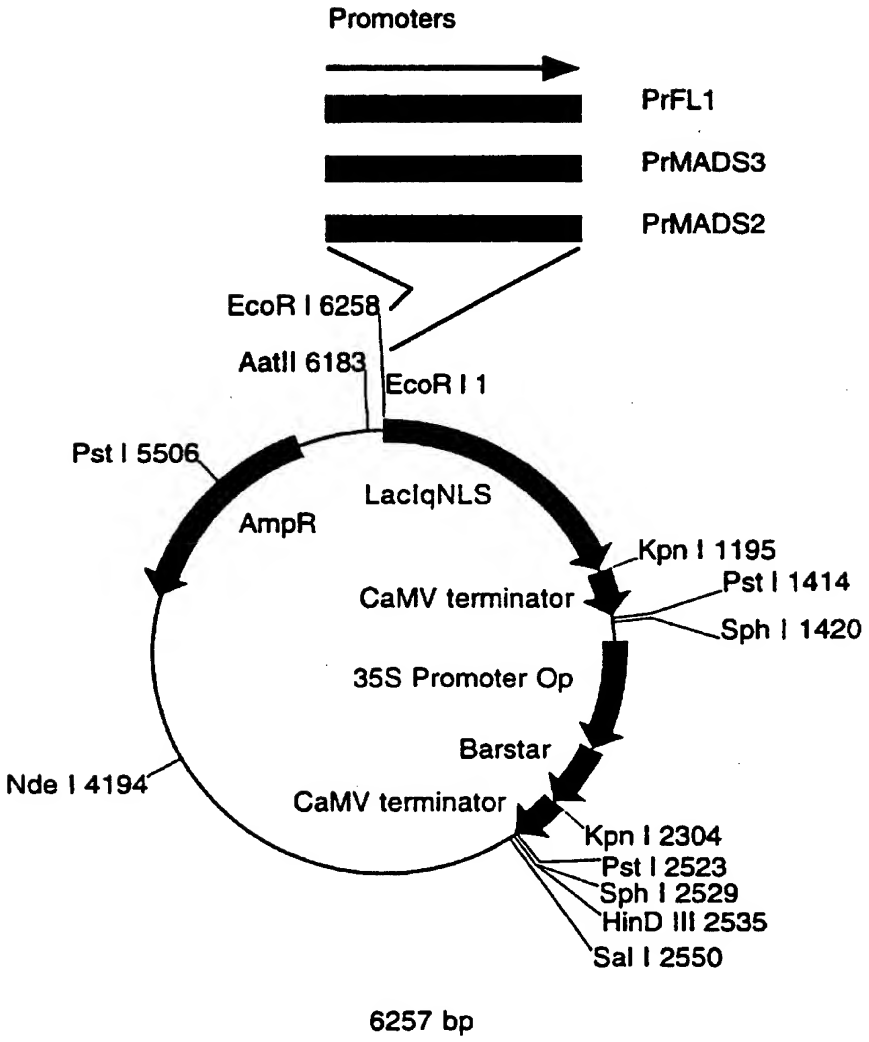

Promoters
PrFL1
PrMADS3
PrMADS2
EcoR I 6258
AatII 6183
EcoR I 1
Pst I 5506
AmpR
LacIqNLS
Kpn I 1195
Pst I 1414
Sph I 1420
CaMV terminator
35S Promoter Op
Barstar
Nde I 4194
CaMV terminator
Kpn I 2304
Pst I 2523
Sph I 2529
HinD III 2535
Sal I 2550
6257 bp

Figure 22

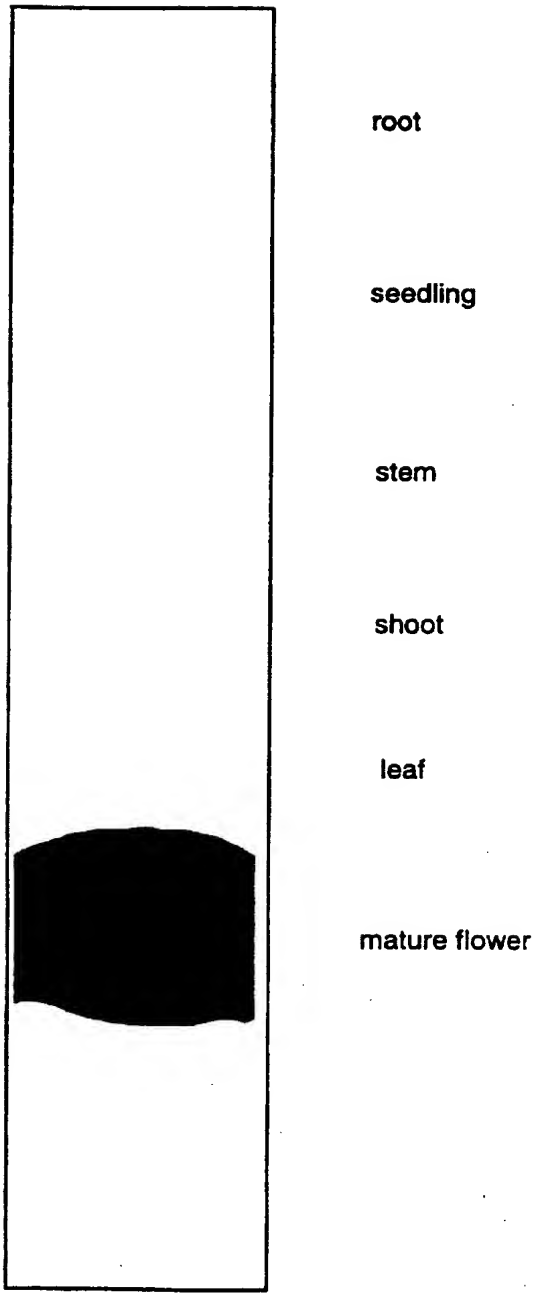


Figure 24

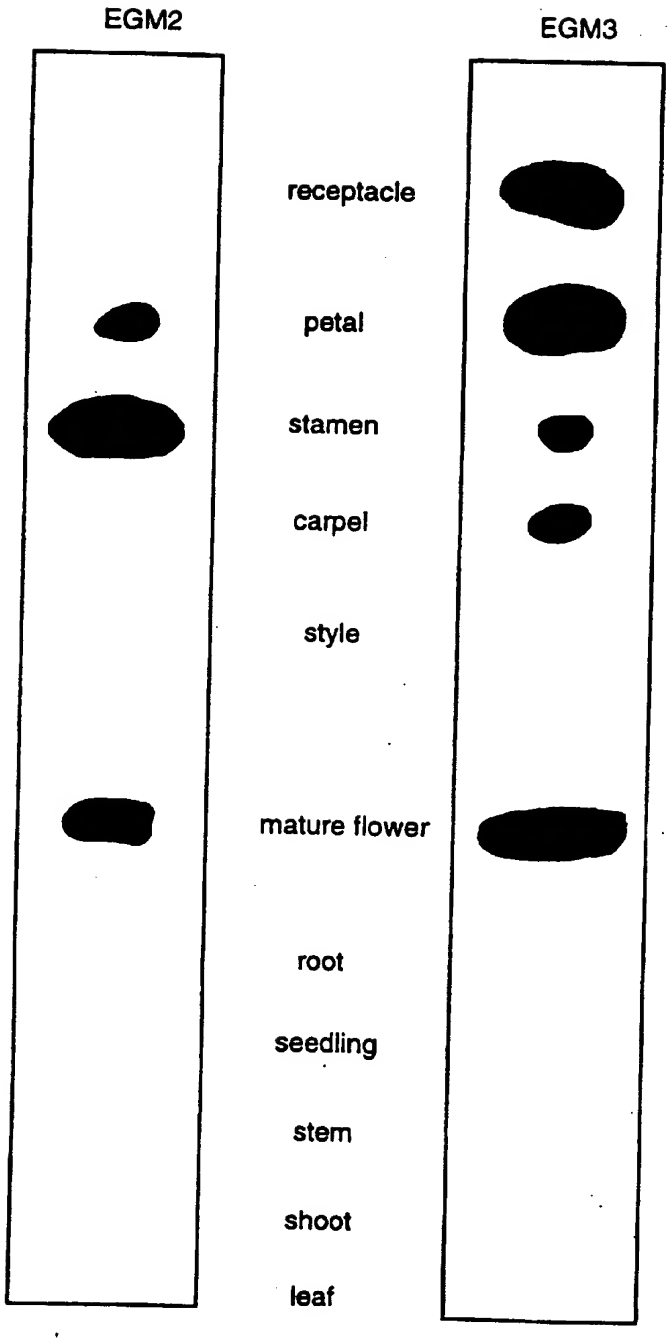

EGM2
EGM3
receptacle
petal
stamen
carpel
style
mature flower
root
seedling
stem
shoot
leaf

Figure 25

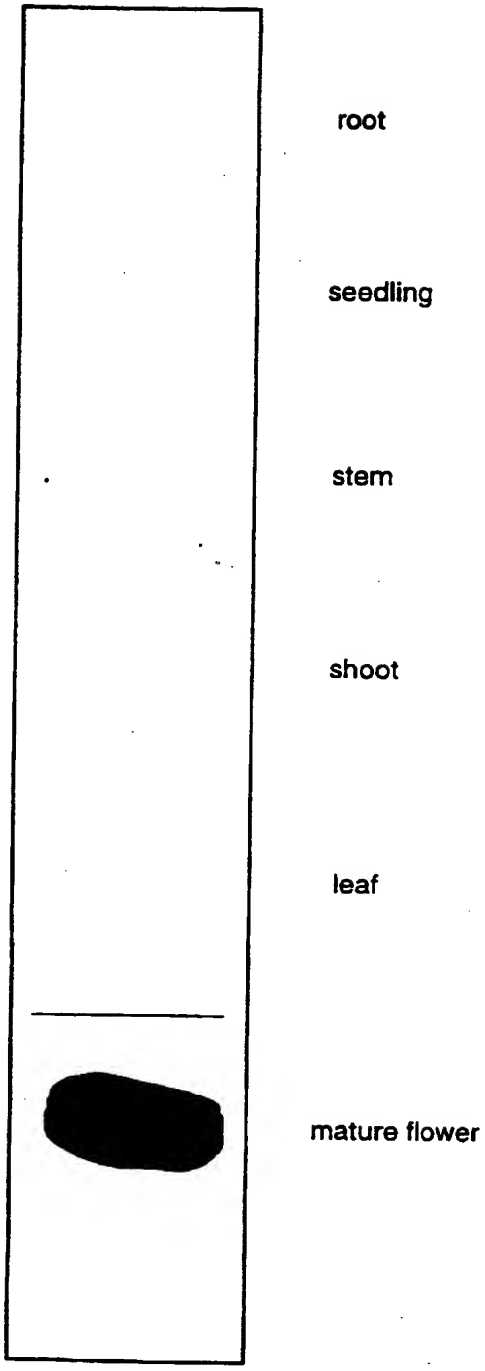


Figure 26

-373

TTTTACTTGGCATAATACATGCACGAACCACTATGCAATCAACCAATTGGGAAGGTTTTTT
TTGTTTTTTTTTTTTTTTT

-293

TGGGGGGGGGTGTTCTGGTTTATTGGGTGGTTCTGGTCTCTTGACATTGTAATTTCCCCT
TAAAATTCCGCTTCATGACA

-213

AAAAGTACATAGAGGGGCTACTATCTTTGTTGACTTTTACCGTGTAGAGGGTTCCTAAGA
GAGAGGGGGGAGGGAAGAGA

-133

GAGACCATGCATAAGTATGGCTAGACACAAGAAAGGTTGCAGAACCGAGAAGAGATCTC
TTTTGAAGAAGTGAACAAATT

-53

TGTGGGATTGTGGGTTGGTGACGAAAACAGCAAAGACAAGAGAAAGGGGAAAGATG

Figure 28

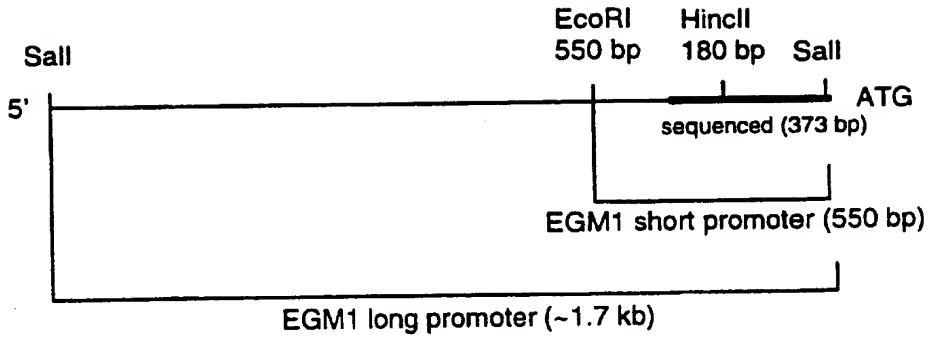

EcoRI
HincII
SalI
550 bp
180 bp
SalI
5'
ATG
sequenced (373 bp)
EGM1 short promoter (550 bp)
EGM1 long promoter (~1.7 kb)

Figure 28A

-1191	A'GCTCC	CGACCGCCAT	GGCCGCGGGG	ATTTAATACG	GACTCCACTA	
-1145	TAGGGAGCTC	GGAATTCCTT	CCTTTGGAAA	AAGGGGGGAA	AAAATCCGTG	
-1095	TCGTTTTTTC	TTTCGTAAAC	AAAAGTCGGA	TGAAATAATA	TGTATTGTTA	
-1045	CTATATATAA	AACAAAAGTG	CTCGTGAAAC	ATTGTCAAAT	ACTATAAGGT	
-995	ATAGACAGGT	TTATGACTCA	CAATTACGAA	GGGGCCTAAA	AGCCGAGAGG	
-945	GAAAAGGTAA	TGCTAGGTAT	AGTACATCTA	GACTATTAAT	TGATATAATA	
-895	AGTAAGTGGA	TCATGTTCAA	TTTCAGATAA	AAATTTCTAT	GTCACGTTGG	
-845	TTTAATATTT	GGAATTTAAT	TATTGGATTT	TATTATATTA	TTCTTTGTCA	
-795	TATTAATTAG	ATTGGTGGAT	ATTTGCCGAT	TCTCATGAGA	GGAAAATTGA	
-745	TCTTTAATTA	ATTTTCAAAA	TACCCCATGGG	CAAGGAAATC	ATAAGGAAAA	
-695	CTCATCTCTT	CCCCAACACG	CAACGCCCAC	ATCGTGCTCT	AGGGTTAAAT	
-645	AGAGATGTGA	TCTCCAAATT	TTTTGTCTGG	GAGCAATGCT	AAAGATGCTA	
-595	AAAAAGTTTA	GCAAGTAGGA	TGACTAACTT	GAAATAGGGC	AGTCAATAAA	
-545	CCAAGTCTAA	CGATCAATTA	CTACATTACA	CGTAATGAGA	AACATTTCGA	
-495	GAAAATCTCT	CTATTTATTG	TGGTTACATA	AGTAGCCTAC	CTATTTGTAA	
-445	TTATGCAAAT	TAACCTAGGA	CGAGCCATAT	ATGCAGTCGA	CATTCATAAG	
-395	GCTTTATAGT	TTGTGCTTCC	GGAAATAGCT	TAGAAAACCA	CATACACTTT	
-345	TCATCAAGAC	TAATCTGACA	GTAAAAAAAG	GAAAAAGAAA	AGAACACCAT	
-295	CAAGACTAAT	GTAATGAAAA	GAAAACCCTA	TTACTAATTT	TGAGAGTTTT	
-245	CCTTTGTAGA	CCCATCACGT	CATCCATCTA	AAACGATGCA	ATGCACACAA	
-195	CTAATGACAA	GGGAACATGT	TAATATACGA	GCAAACCTAA	CCAGGTTTCC	
-145	CCTTCCTTCA	TTAGAGTATA	ATAACTGTTC	CCTTTTCTAC	CTCCTTTTTG	
-95	TACCCTAGCT	CTCGAGCTCC	TTAAGAAAGT	TTGCTCGGCT	CTTAACCAGG	
-45	ACTAGAAAAG	ATCAGAGAGA	GAAAGAGAGA	AAGAGAGACA	GAGATATGGG	
+5	AAGAGGG					

Figure 29

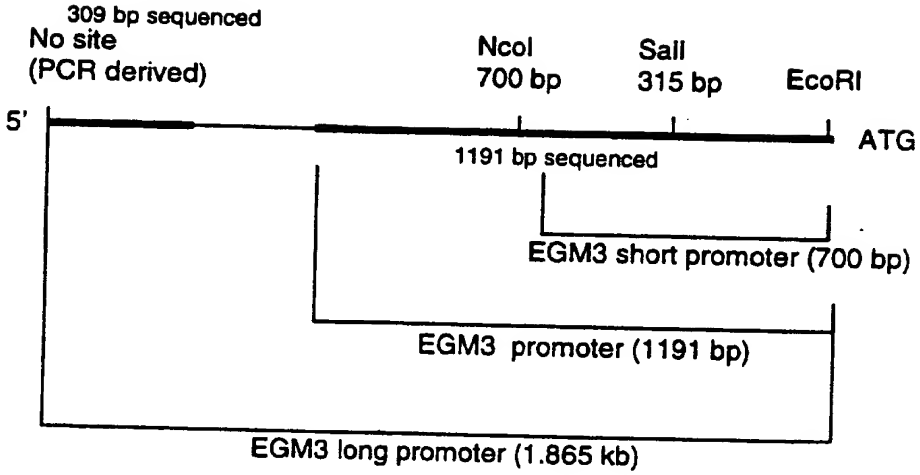

309 bp sequenced
No site
(PCR derived)
NcoI
700 bp
SalI
315 bp
EcoRI
5'
ATG
1191 bp sequenced
EGM3 short promoter (700 bp)
EGM3 promoter (1191 bp)
EGM3 long promoter (1.865 kb)

Figure 29A

-1865

TAGCTCTCGAGCTCCTTAAGAAAGTTTGCTCGGCTCTTAACCAGGACTAG

-1815

AAAAGATCAGGGAGAGAAAGAGAGAAAGAGAGACAGGGATATGGGAAGAG

-1765

GGAAAGTATAGCTGAAGAGGATAGAGAACAAAATCAACAGGCAAGTAACA

-1715

TTTGCGAAGAGAAGAAATGGGCTTCTCAAGAAAGCTTATGAGCTCTCTGT

-1665

TCTCTGTGATGCTGAAATCACTAGTGCGGCCGCCTGCAGGTCGACCATAT

-1615

GGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTC

-1565

ACCTAAATA -1556

Figure 29B

-940

ATAGAGCAGTACACTTTAAGGGAGCACGCACCCCAATTTGATATAAGGTACAAAGTTTTG
TTCCAAGATGTGGGTGACAT

-860

CATTAAATGAGATTTACCATCTTTCAACAGGTTCAAAGGGTACCCTGTACTATAAAGCCCA
CGAAGCTATTTGTATGTTA

-780

AAAAAAAATTCACATAGACTAGGTACGATCGAAAAGCTCATTAAGGAGCTGCCAACGAAA
AGTTCACTTTGTTAACCTCT

-700

CCTCTTATCTTATATTCAATAAGAATATGCACATCGGTTGTGATTTGATGCTCAATTGAACT
TGACATTTGGTTATTGGT

-620

TCTACCCACTTAAACCCTAAGCAAAAAGGCCAGAAATGTGCACACATTATTCATCAAGCC
GGGTGCCACGTGTCCAGTAA

-540

GACAAACTAAATAAGGAGAAAATACTGGTAAGGAAGCCTATGTTGTGTATGTAAGATACC
CAATGATAGTCCGTGGTTTT

-460

GATAAGTTCAAACTTAACATTTGAAAAAGGGACAATATGACGAAGCTGCAGTTAGAACAG
GAGAACACCCCAACCCGAGA

-380

AAATATCCGGTCGTTTAATATCAATAGCTCACGTCAGTGCAGGATGTCTGAAAAYGACCG
ATGGTCACACGCGCGTGACA

-300

TGGAAGAGAAGAACCAATAACCAGAAAGCCCTAATAATGAACAGTAGACTTTAGCATGA
AACCTCTCACCTTTTCTTTTG

-220

TGCCAAGAAAGTCGCAGTTCCCCTTAACCCTAAATGGAATGTTGTGTCTTTACTTCTGTAC
CTCTCCTTTTTTTCAGCCT

-140

CTCTCTCTCTCTCTCTCTCTCTCTCAGTCTCAGCTATTCAGCTATTCTGCTCCTCAGCTT
TCATTTGCAAACAAGAGC

-60

AGATAAGGAAGATAATACGAGAGAGAACAAGAGAGAAAGAGAAGGCCCGGAAAAAGAA
AGATCGGTGCAC

(mutated ATG)

Figure 30

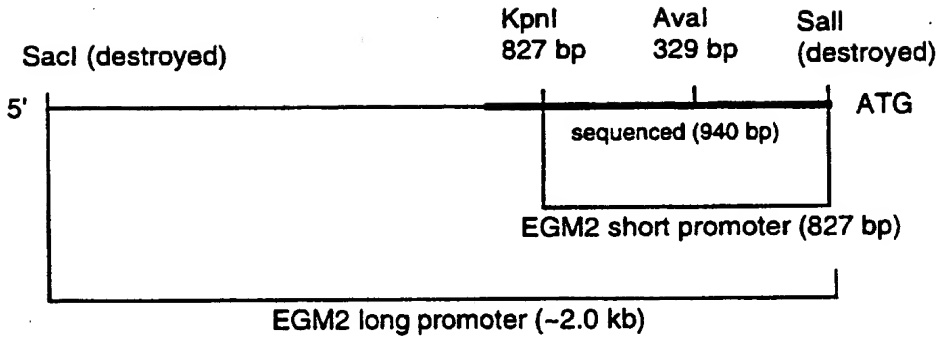

KpnI
827 bp
AvaI
329 bp
SalI
(destroyed)
SacI (destroyed)
5'
ATG
sequenced (940 bp)
EGM2 short promoter (827 bp)
EGM2 long promoter (~2.0 kb)

Figure 30A

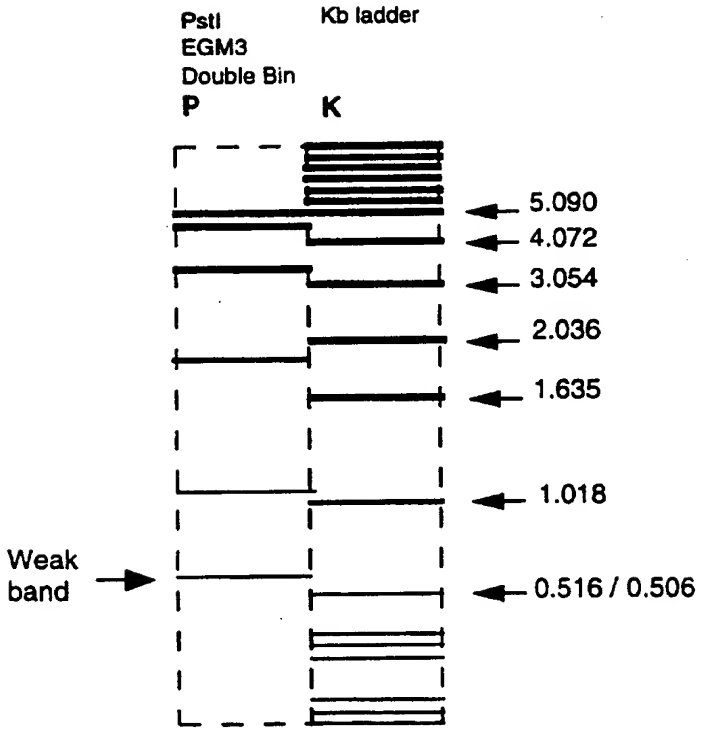

PstI
EGM3
Double Bin
P
Kb ladder
K
5.090
4.072
3.054
2.036
1.635
1.018
Weak
band
0.516 / 0.506

Figure 31

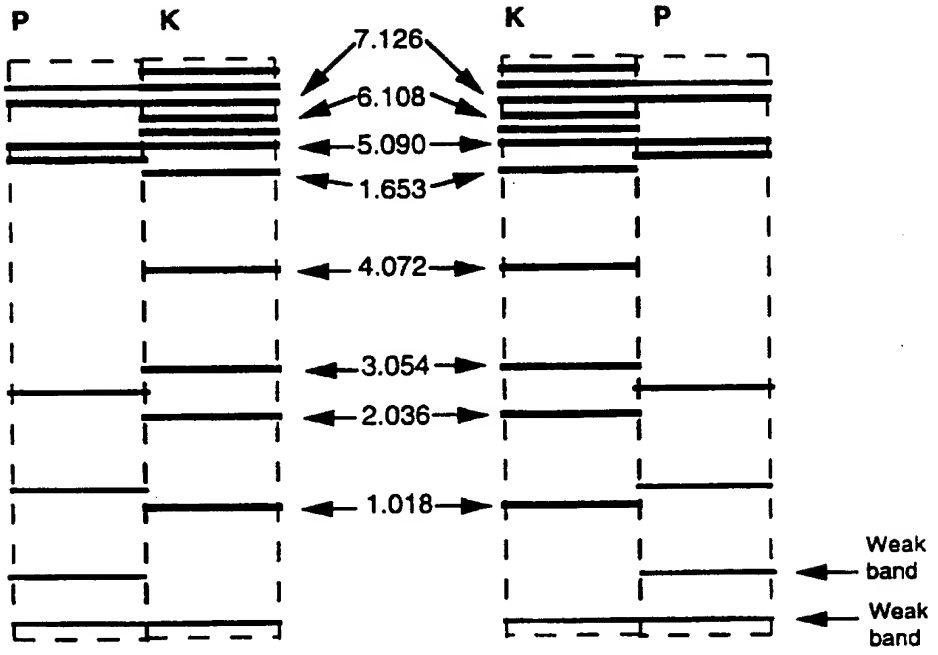

P
K
K
P
7.126
6.108
5.090
1.653
4.072
3.054
2.036
1.018
Weak band
Weak band

Figure 32

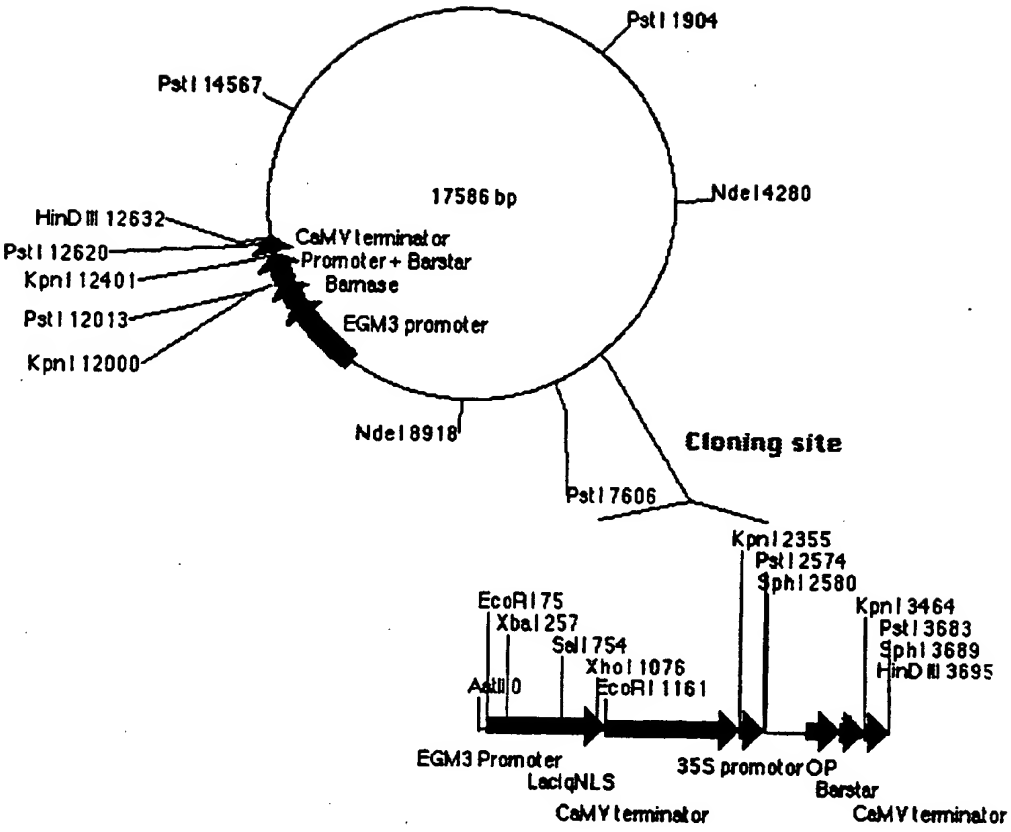

PstI 1904
PstI 14567
17586 bp
NdeI 4280
HinDIII 12632
PstI 12620
KpnI 12401
PstI 12013
KpnI 12000
CaMV terminator
Promoter + Barstar
Barnase
EGM3 promoter
NdeI 8918
Cloning site
PstI 7606
KpnI 2355
PstI 2574
SphI 2580
KpnI 3464
PstI 3683
SphI 3689
HinDIII 3695
EcoRI 75
XbaI 257
SalI 754
XhoI 1076
AatII 0
EcoRI 1161
EGM3 Promoter
LacIqNLS
CaMV terminator
35S promotor OP
Barstar
CaMV terminator

Figure 33

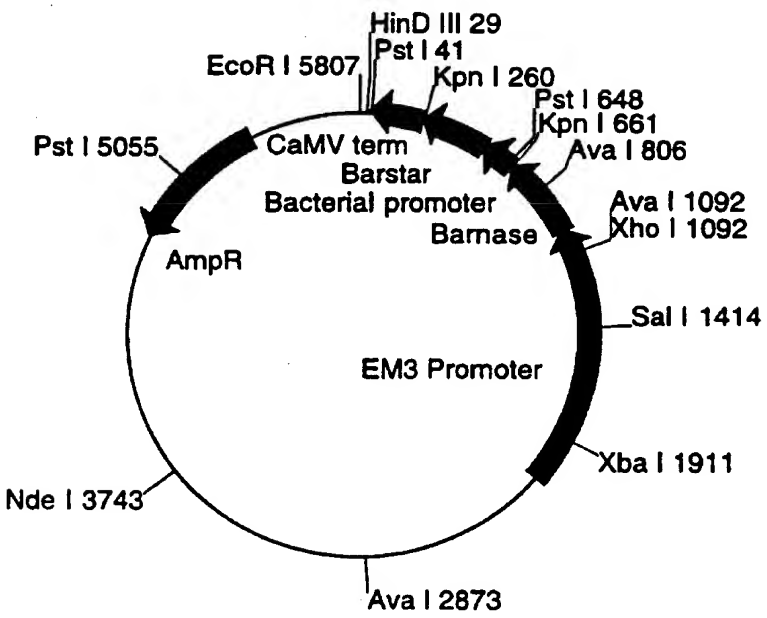

HinD III 29
Pst I 41
EcoR I 5807
Kpn I 260
Pst I 648
Kpn I 661
Pst I 5055
CaMV term
Ava I 806
Barstar
Bacterial promoter
Ava I 1092
Barnase
Xho I 1092
AmpR
Sal I 1414
EM3 Promoter
Xba I 1911
Nde I 3743
Ava I 2873
5809 bp

Figure 34

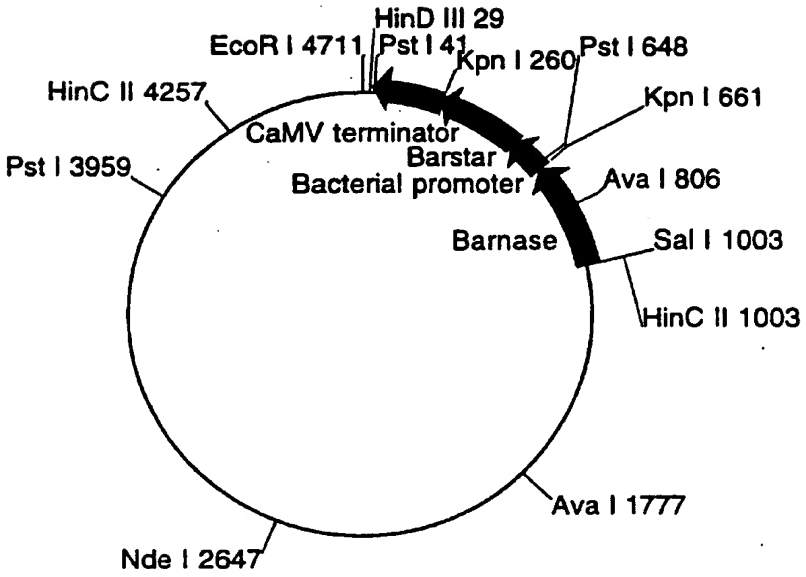
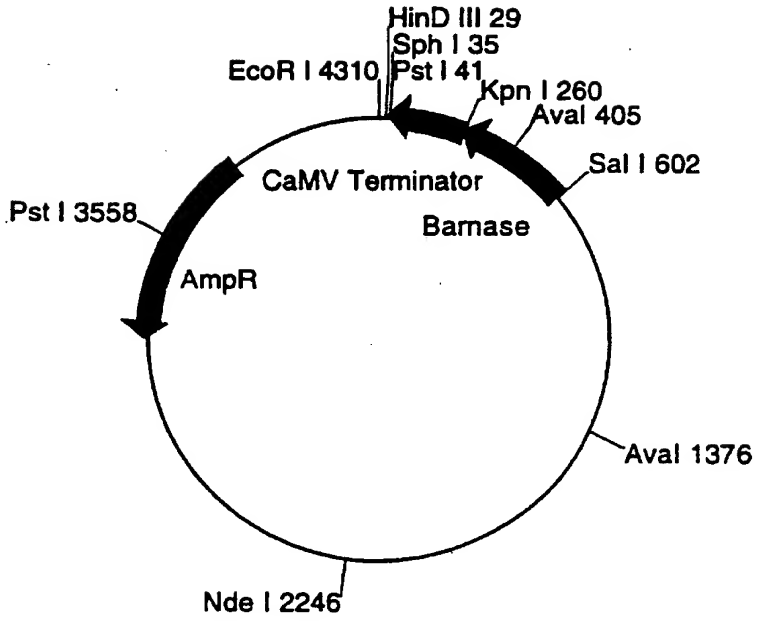

HinD III 29
EcoR I 4711
Pst I 41
Kpn I 260
Pst I 648
HinC II 4257
Kpn I 661
CaMV terminator
Barstar
Pst I 3959
Bacterial promoter
Ava I 806
Barnase
Sal I 1003
HinC II 1003
Ava I 1777
Nde I 2647
4713 bp

Figure 35



4312 bp

Figure 36

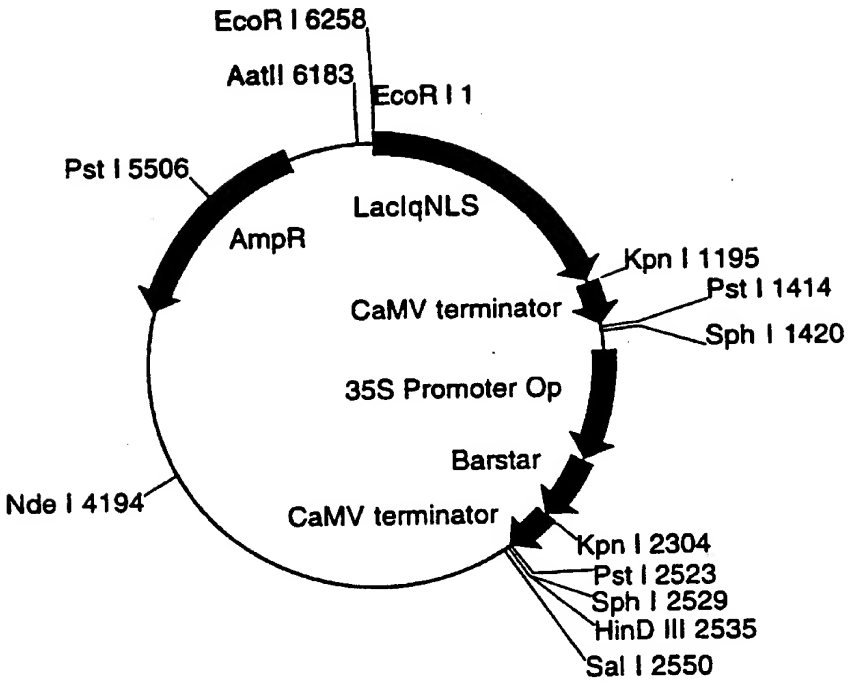

EcoR I 6258
AatII 6183
EcoR I 1
Pst I 5506
LacIqNLS
AmpR
Kpn I 1195
Pst I 1414
CaMV terminator
Sph I 1420
35S Promoter Op
Barstar
Nde I 4194
CaMV terminator
Kpn I 2304
Pst I 2523
Sph I 2529
HinD III 2535
Sal I 2550
6257 bp

Figure 37

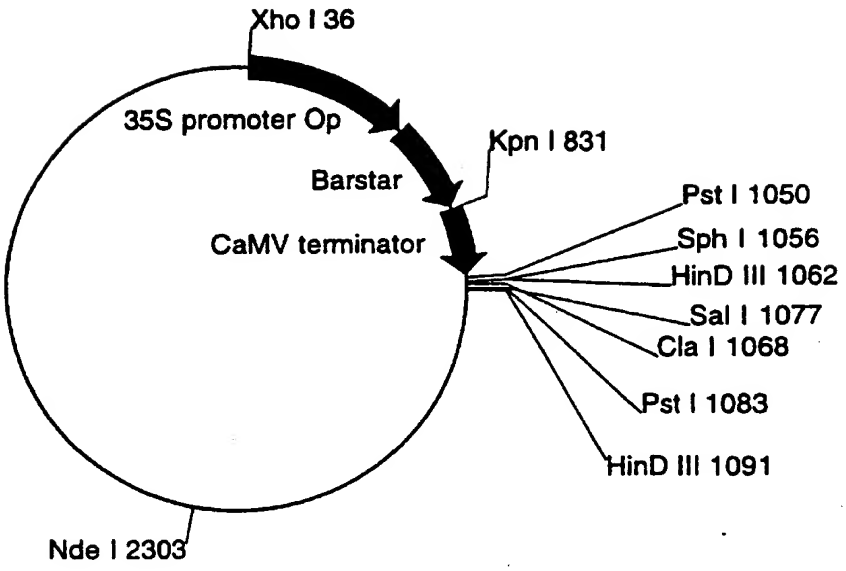

Xho I 36
35S promoter Op
Kpn I 831
Barstar
Pst I 1050
CaMV terminator
Sph I 1056
HinD III 1062
Sal I 1077
Cla I 1068
Pst I 1083
HinD III 1091
Nde I 2303
4330 bp

Figure 38

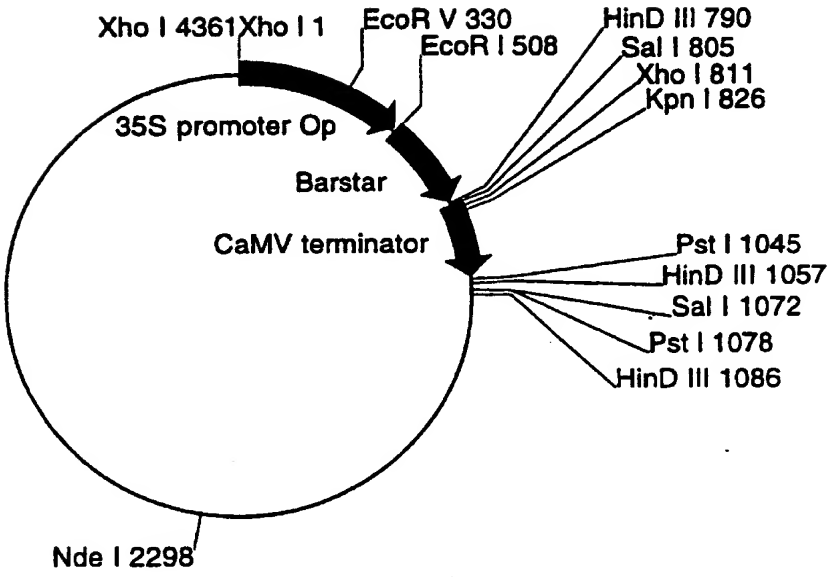

Xho I 4361
Xho I 1
EcoR V 330
EcoR I 508
HinD III 790
Sal I 805
Xho I 811
Kpn I 826
35S promoter Op
Barstar
CaMV terminator
Pst I 1045
HinD III 1057
Sal I 1072
Pst I 1078
HinD III 1086
Nde I 2298
4360 bp

Figure 39

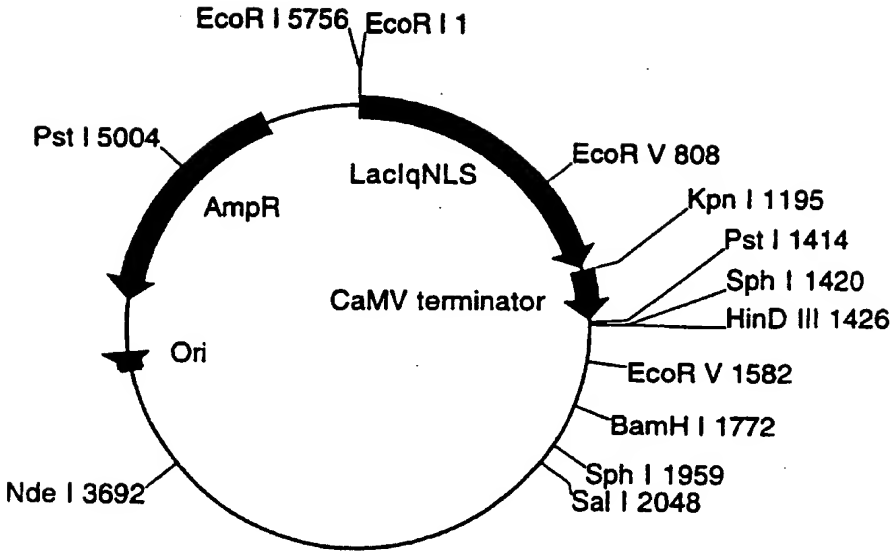

EcoR I 5756
EcoR I 1
Pst I 5004
LacIqNLS
EcoR V 808
AmpR
Kpn I 1195
Pst I 1414
Sph I 1420
CaMV terminator
HinD III 1426
Ori
EcoR V 1582
BamH I 1772
Sph I 1959
Nde I 3692
Sal I 2048
5755 bp

Figure 40

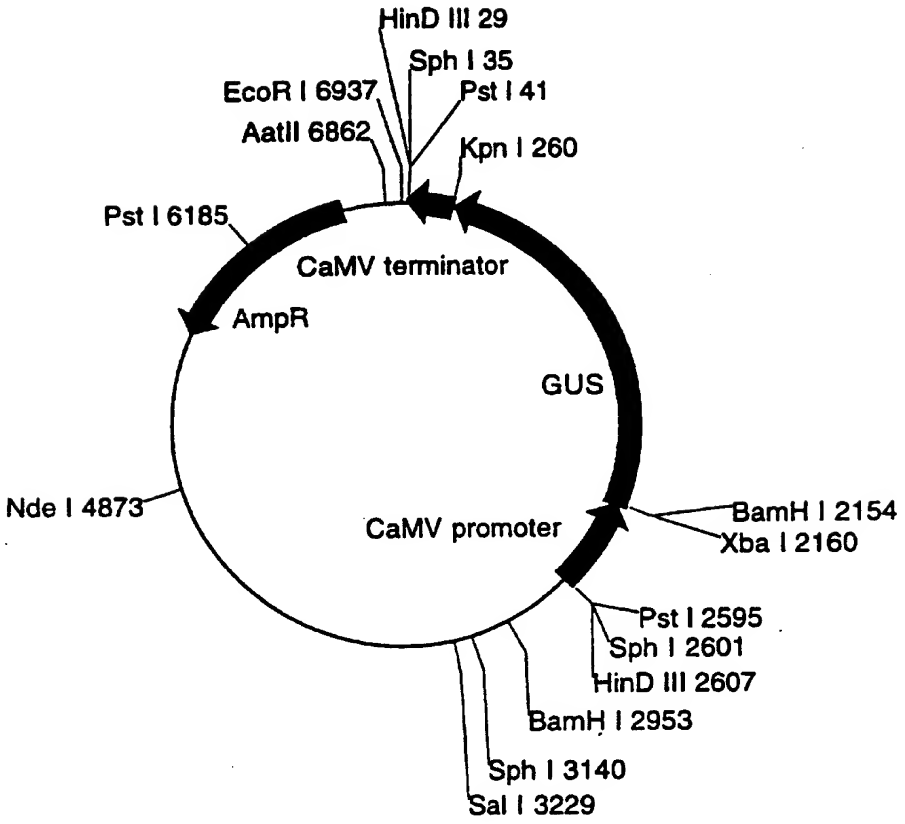

HinD III 29
Sph I 35
EcoR I 6937
Pst I 41
AatII 6862
Kpn I 260
Pst I 6185
CaMV terminator
AmpR
GUS
Nde I 4873
CaMV promoter
BamH I 2154
Xba I 2160
Pst I 2595
Sph I 2601
HinD III 2607
BamH I 2953
Sph I 3140
Sal I 3229
6939 bp

Figure 41

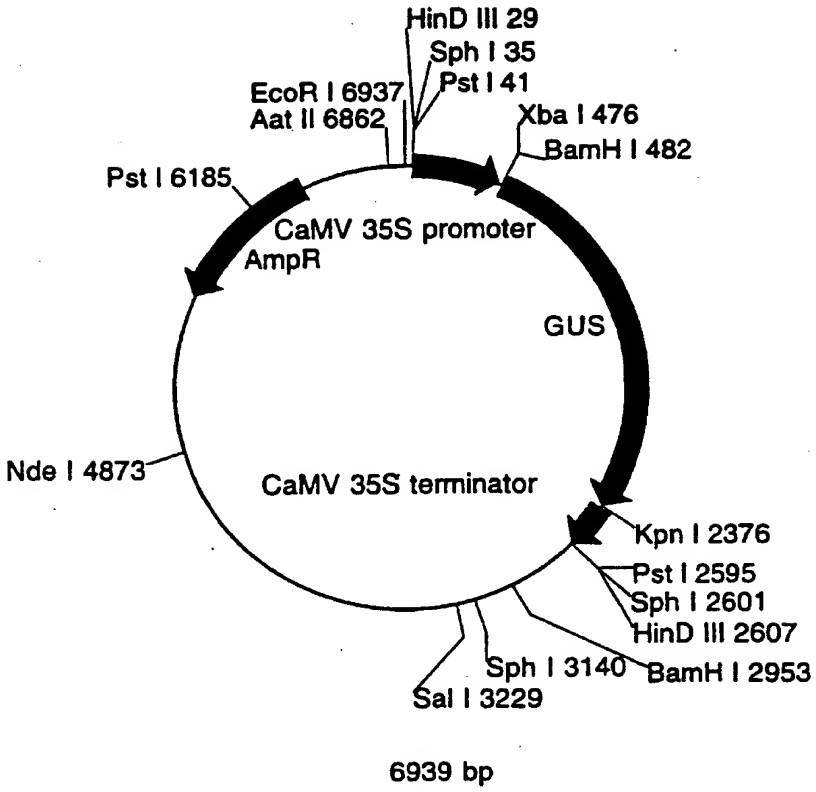

HinD III 29
Sph I 35
Pst I 41
EcoR I 6937
Aat II 6862
Xba I 476
BamH I 482
Pst I 6185
CaMV 35S promoter
AmpR
GUS
Nde I 4873
CaMV 35S terminator
Kpn I 2376
Pst I 2595
Sph I 2601
HinD III 2607
Sph I 3140
BamH I 2953
Sal I 3229
6939 bp

Figure 42

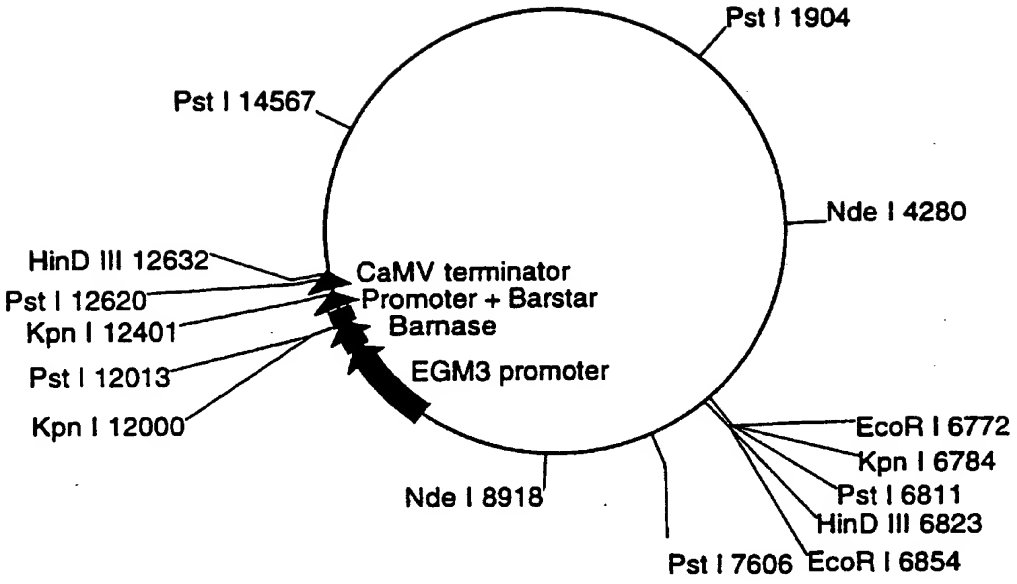

Pst I 1904
Pst I 14567
Nde I 4280
HinD III 12632
Pst I 12620
Kpn I 12401
Pst I 12013
Kpn I 12000
CaMV terminator
Promoter + Barstar
Barnase
EGM3 promoter
EcoR I 6772
Kpn I 6784
Pst I 6811
HinD III 6823
EcoR I 6854
Pst I 7606
Nde I 8918
17586 bp

Figure 43

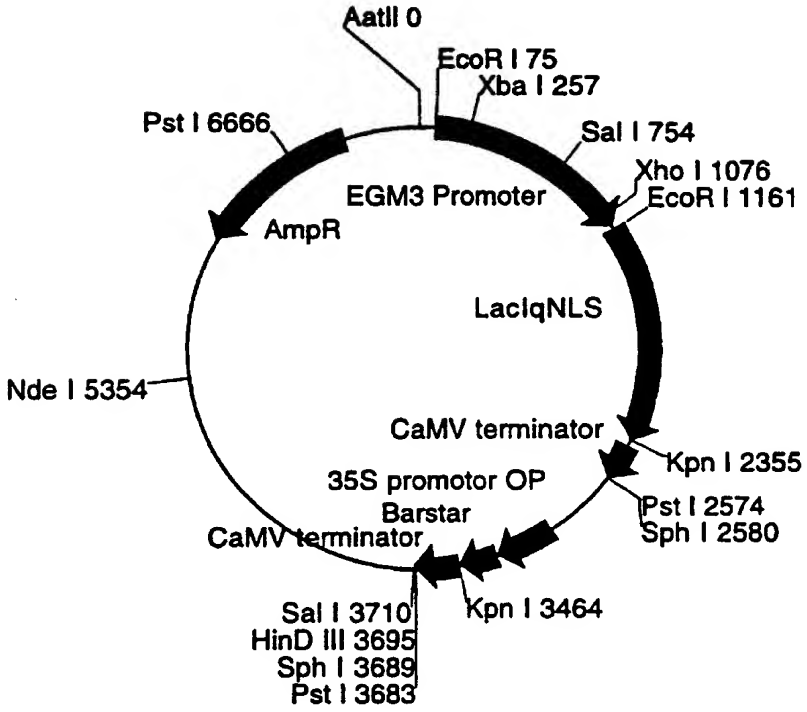

AatII 0
EcoR I 75
Xba I 257
Sal I 754
Xho I 1076
EcoR I 1161
Pst I 6666
EGM3 Promoter
AmpR
LacIqNLS
Nde I 5354
CaMV terminator
Kpn I 2355
35S promotor OP
Barstar
Pst I 2574
Sph I 2580
CaMV terminator
Kpn I 3464
Sal I 3710
HinD III 3695
Sph I 3689
Pst I 3683
7343 bp

Figure 44

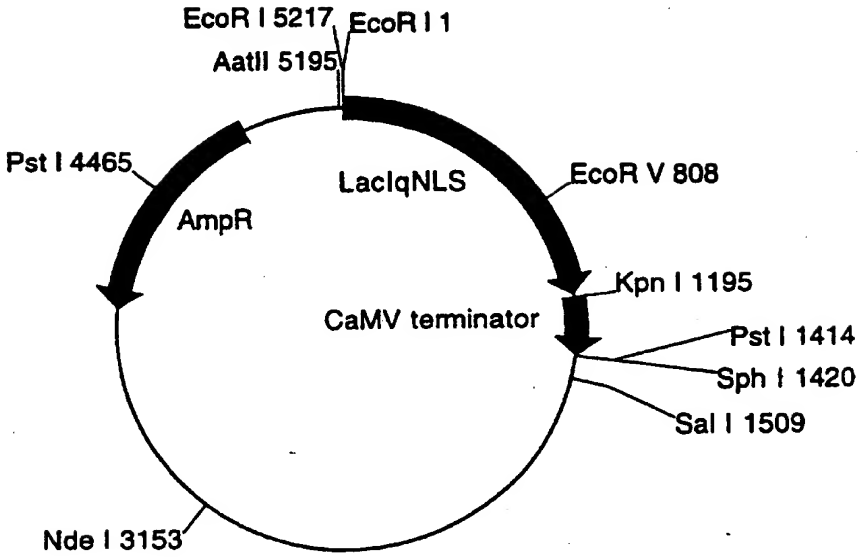

EcoR I 5217
EcoR I 1
AatII 5195
Pst I 4465
LacIqNLS
EcoR V 808
AmpR
Kpn I 1195
CaMV terminator
Pst I 1414
Sph I 1420
Sal I 1509
Nde I 3153
5216 bp

Figure 45

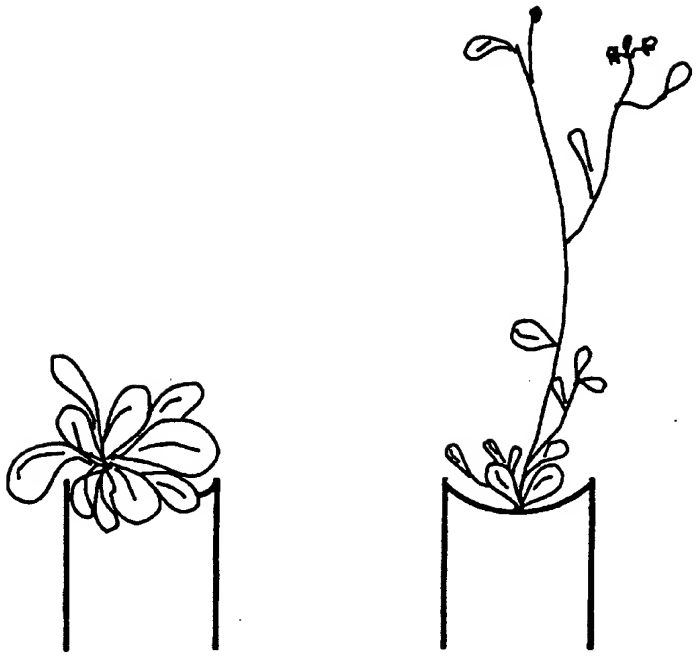


Figure 46

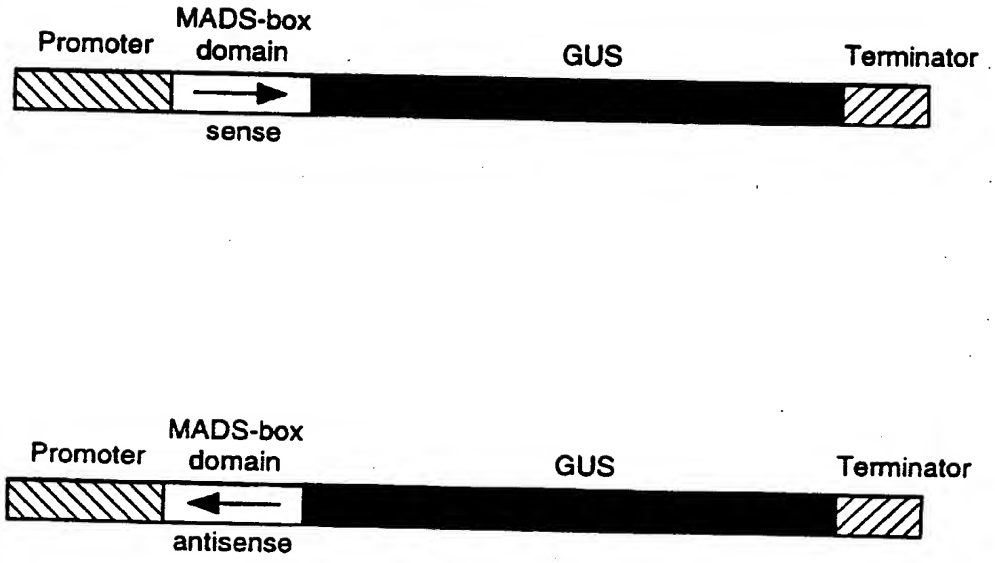

Promoter
MADS-box domain
GUS
Terminator
sense
Promoter
MADS-box domain
GUS
Terminator
antisense

Figure 47

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00625

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 15/82, 15/84

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC C12N 15/82, 15/84
CHEMICAL ABSTRACTS

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
(SEE ATTACHED)-ATTACHMENT 1

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X, Y	AU 17132/97 A (FORBIO RESEARCH PTY. LTD.) 21 August 1997. See whole document in particular. See plasmids disclosed.	1 to 29
X, Y	AU 86224/91 B (FB INVESTMENTSPTY.LTD) 15 April 1992. See examples and claims.	1 to 29
X, Y	Strauss, S.H. et al. (1995) Genetic engineering of reproductive sterility in forest trees, *Moleculor Breeding, vol.* 1, 5-26.	

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
31 October 1997

Date of mailing of the international search report
10 NOV 1997

Name and mailing address of the ISA/AU
AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION
PO BOX 200
WODEN ACT 2606
AUSTRALIA Facsimile No.: (02) 6285 3929

Authorized officer
D. HENNESSY
Telephone No.: (02) 6283 2255

INTERNATIONAL SEARCH REPORT

ernational Applicati n N .
PCT/AU 97/00625

C (Continuation) **DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, Y	WO 94/25613 A (CORNELL RESEARCH FOUNDATION, INC.) 10 November 1994 See whole document	1 to 5
X, Y	WO 94/00582 A (CENTERE FOR PLANT BREEDING AND REPRODUCTION RESEARCH) 6 January 1994. See whole document	1 to 5, 9
P, X, Y	Columbo, L. et al. (1997) downregulation of Ovule-specific MADS BOX genes from Petunia results in maternally controlled defects in seed development, *The Plant Cell*, vol. 9, 703-715. See the plasmids used in particalar.	1 to 29
X, Y	Thorsness, M. K et al. (1993) Genetic ablation of floral cells in arabidopsis, *the plant cell*, vol. 5, 253-261. See the plasmids used in particular.	1 to 5, 9
X, Y	Kandasamy, M.K. et al (1993) Ablation of papillar cell function in brassica flowers results in the loss of stigma receptivity to pollination. *The Plant cell*, vol 5, 263-275-See whole articles	

INTERNATIONAL SEARCH REPORT

...rnational Application No.
PCT/AU 97/00625

B x ATTACHMENT 1

Databases consulted: Derwent 'WPAT', CHEMICAL ABSTRACTS -
FILE: CA
AGRICOLA

Keywords: PROMOTER, FERTIL:, STERIL:, BARNASE, BARSTAR, REPRODUCT:, OVAR: OR STAMENT# OR ANTHER# OR CARPEL # OR CONE# OR FRUIT: (W) BOD:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.
PCT/AU 97/00625

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
AU	17132/97						
AU	86224/91	BR	9106926	EP	550543	CA	2092549
		WO	9205257				
WO	94/25613	AU	68191/94				
WO	94/00582	EP	672155				

END OF ANNEX